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Review

Molecular Breeding for Improved Second Generation Bioenergy Crops

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There is increasing urgency to develop and deploy sustainable sources of energy to reduce our global dependency on finite, high-carbon fossil fuels. Lignocellulosic feedstocks, used in power and liquid fuel generation, are valuable sources of non-food plant biomass. They are cultivated with minimal inputs on marginal or degraded lands to prevent competition with arable agriculture and offer significant potential for sustainable intensification (the improvement of yield without the necessity for additional inputs) through advanced molecular breeding. This article explores progress made in next generation sequencing, advanced genotyping, association genetics, and genetic modification in second generation bioenergy production. Using poplar as an exemplar where most progress has been made, a suite of target traits is also identified giving insight into possible routes for crop improvement and deployment in the immediate future.

Molecular Breeding for Sustainable Intensification

In the past decade advanced breeding techniques have been increasingly employed to enhance commercially important traits in staple crops and livestock as food producers seek to improve the yield, economy, resilience, and environmental sustainability of their products [1]. Next generation sequencing (NGS), high-throughput genotyping, and **molecular breeding** (see Glossary) methodologies such as marker assisted selection (MAS), **genomic selection** (GS), and genetic modification (GM) have been applied in a significant number of important species. These include cereals such as rice (*Oryza sativa*) [2], maize (*Zea mays*) [3], and barley (*Hordeum vulgare*) [4]; other crops including potato (*Solanum tuberosum*) [5], apple (*Malus domestica*) [6], and soybean (*Glycine max*) [7]; and domestic livestock species including cattle [8] and pigs [9]. In total more than 100 plant genomes have been sequenced since 2000 [10] as costs for sequencing technology have plummeted and instrument capacity increased millionfold [11]. Targeted phenotypes vary depending upon the breeding priorities for a given species but tend to be broadly focussed on disease resistance [4]; maximising yields [7]; improving nutritional quality [3]; reducing waste [9], or inducing tolerance to more challenging environmental conditions such as drought and salt stress [12]. Many of these breeding approaches may enable us to realise the 'more from less' paradigm suggested as part of the sustainable intensification of crop production. Here we take the Royal Society's definition of sustainable intensification as a system, 'in which yields are increased without adverse environmental impact and without the cultivation of more land' and suggest that primary productivity should be refocused towards these goals [13]. There remains considerable potential to better exploit genetic resources where the development of new and novel crops is focussed away from yield *per se* and towards improved quality of product with reduced inputs and waste. For **bioenergy** crops, that have hardly been selected and bred in the past, this provides a framework to rapidly harness the power of new DNA

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Second generation bioenergy feedstocks cultivated from non-food crops on marginal lands are widely held as a promising source of renewable and sustainable energy to help displace fossil fuels.

A substantial yield gap exists for key bioenergy crops and this has held back their successful commercial deployment for reasons of economy and sustainability.

Affordable advanced molecular breeding techniques have driven progress in food crops in recent years as next generation sequencing has dramatically reduced costs.

Traits of interest for improvement in bioenergy include biomass yield, feedstock quality, drought tolerance, and pest resistance.

Genotyping by sequencing, association genetics, and genetic modification are now beginning to be applied in second generation bioenergy species with a view to addressing these breeding targets.

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technologies to deliver higher, more sustainable yields that are of wide value to society. Such crops include perennials trees including poplar (*Populus*) and willow (*Salix*) and grasses such as *Miscanthus* (*Miscanthus sinensis*, *Miscanthus sacchariflorus*, *Miscanthus × giganteus*), *Arundo donax*, and switchgrass (*Panicum virgatum*); grown for their **lignocellulosic** biomass.

The progress of bioenergy has been hampered by controversy surrounding first generation biofuels derived from food crops, with public opposition over their suggested impact on food security and debate as to the true extent of the benefit they provide over conventional fossil fuels. Second generation lignocellulosics are considered as promising candidates for the production of sustainable, cost-effective bioenergy feedstocks, but in contrast to food crops, a relatively short time and limited effort has been focussed on their breeding and improvement with very little commercial deployment to date despite the clear advantages they confer over the first generation. The key question is whether the opportunity provided by new DNA technologies can help enable the accelerated development of better lignocellulosic crops. Such crops require research effort to maximise their inherent potential for competitive yields, reduced greenhouse gas (GHG) emissions, and favourable energy balances relative to the first generation bioenergy and fossil fuels it is hoped they will replace [14]. Thus the central aim for bioenergy breeding must be sustainable yield intensification, that is, increasing biomass production per land unit area without environmental degradation or increased agronomic inputs.

The Yield Gap in Second Generation Bioenergy

The yield gap may be considered as the difference between potential and actual yield, where potential yield (Yp) is defined as the yield obtained if the full genetic potential of a crop is realised under optimal conditions in which water and nutrients are non-limiting and biotic stresses are controlled (often only seen in experimental conditions). Actual yield (Ya), by contrast, is the average yield obtained in the field and the yield gap is calculated as the difference between Yp and Ya [15]. Yield gap analyses are commonly employed for food crops including rice [16], maize [17], and cassava (*Manihot esculenta*) [18] and consider the impact of pests and disease, water availability, and soil nutrients on crop performance in a given climate or location. As such, they are considered important for the sustainable intensification of agriculture by highlighting which crops and regions have the greatest potential for increased productivity and identifying research priorities for crop improvement [15].

Figure 1 illustrates the range of biomass yields obtained in published field trials for poplar, willow, and *Miscanthus*. Unsurprisingly the highest yields are generally achieved in trials with supplemental irrigation and/or fertiliser application and the breadth of reported yields is suggestive of a significant yield gap for these key lignocellulosic bioenergy crops. While potential yields are rarely achieved, the possibility exists for targeted molecular approaches to help overcome this gap in a timely manner by addressing traits that can drive the sustainable intensification of second generation biomass. These data suggest a yield gap of approximately 15 tonnes ha^{-1} year^{-1}, providing a significant gap that may be addressed using molecular breeding for the accelerated deployment of these relatively new crops.

Key Traits for the Sustainable Intensification of Bioenergy

To pursue the sustainable intensification of biomass, research must target traits that can deliver increased yields while minimising the need for agronomic inputs, that is, supplementary irrigation, fertilisation, and pesticide application. Performance must also be consistent, with perennial bioenergy crops able to provide reliably high yields over multiple harvest cycles in the face of variable and changing climatic conditions. Although grain yield and not biomass yield is key to food crop improvement, many of the traits that represent breeding priorities for bioenergy are also relevant to food (pest and disease resistance, drought and salinity tolerance, and nitrogen and water use efficiencies). These traits underpin the delivery of sustainable intensification for

Glossary

Bioenergy: renewable energy derived from biomass, generally plant material or organic waste. Examples of bioenergy include liquid biofuels for transport, biomass for combustion or biogas.

Genotyping-by-sequencing (GBS): the resequencing of multiple genomes of a species to discover large numbers of SNPs for GWAS or genomic selection.

Genomic selection (GS): requires phenotyping in a large training population followed by assignment of breeding values for individuals based solely on genotyped, trait-associated markers. Unlike in GWAS the effects of all genetic markers are estimated simultaneously.

Genome editing: the use of engineered nucleases to execute precise insertions, alterations, or deletions from the target genome. The CRISPR/Cas system is a powerful new tool for genome editing in eukaryotes.

Genome-wide association study (GWAS): a forward genetic approach; GWAS uses DNA markers across the genome of a species and seeks statistical associations between markers and traits of interest, which can inform candidate gene identification and marker assisted breeding.

Lignocellulosics: second generation bioenergy feedstocks derived from plants and comprising the glucose polymer cellulose and associated phenolic polymer lignin that constitute the majority of the secondary cell wall in such species.

Molecular breeding: the application of molecular biology tools especially DNA markers to plant or animal breeding. These include QTL mapping, gene discovery, marker assisted selection (MAS), genomic selection (GS), and genetic modification (GM).

Single nucleotide polymorphism (SNP): a DNA sequence variation caused when a single nucleotide (A, T, C, or G) varies at a given position in the genome between members of the same species.

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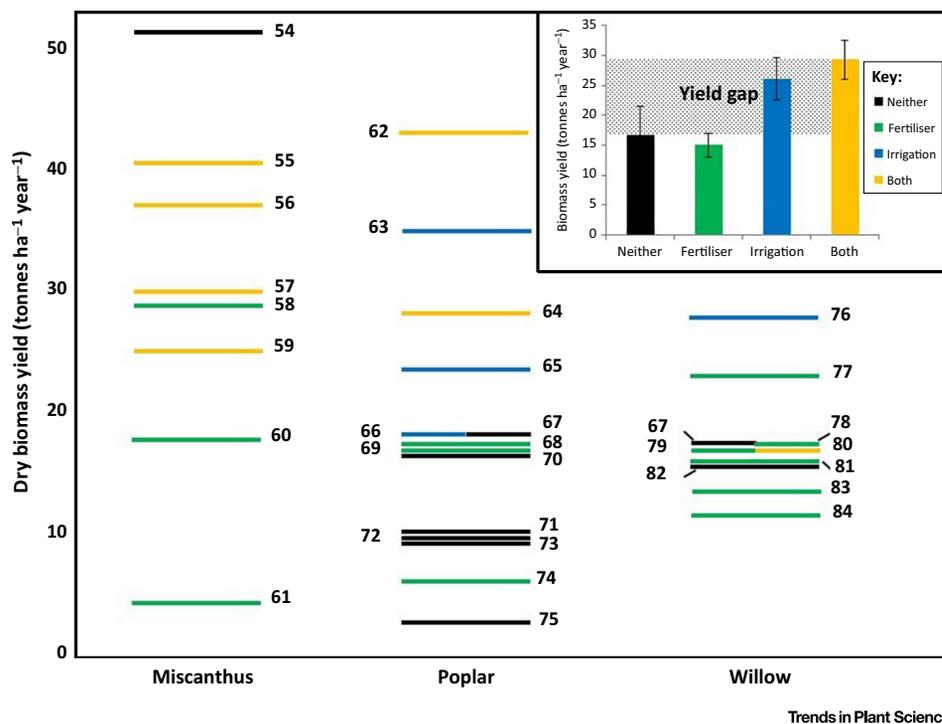
Figure 1. Reported Biomass Yields Reveal a Yield Gap for Second Generation Bioenergy. The second generation bioenergy feedstocks poplar, willow, and *Miscanthus* have shown wide variation in their biomass yields. Each bar represents a single study and where more than one value is reported in a publication those given here are the maximum reported oven-dry biomass yields (tonnes ha^{-1} year^{-1}) for the best performing sites, genotypes, and years or coppice cycles within each study. Numerical citations adjacent to each bar correspond to a single published field trial [54–84]. Poplar and *Miscanthus* show a greater range of values than willow with larger maximum biomass yields reported. The inset bar chart displays the mean yield and standard error for all the trials shown, pooled across feedstocks for each management practice. This reveals a clear and expected trend towards greater yields in trials with supplemental fertiliser and irrigation with a single exception in the case of the highest yielding *Miscanthus* trial; which reported remarkably high yields and solar conversion efficiencies in nutritionally rich, silt agricultural soils in Illinois, USA [54]. The yield gap may in reality be greater than indicated by the shaded region of the chart as commercial yields may fall short of those reported in experimental plots and trials.

bioenergy but sit alongside bioenergy-specific traits such as feedstock composition and conversion efficiency, which are important for tailoring biomass to the requirements of a particular process, be it bioethanol production or the development of pyrolysis oil – thus increasing the productivity and profitability of the fuel chain as a whole [19]. Table 1 takes poplar as an exemplar species, where most progress has been made and provides an extensive list of traits shown to be of value for its development related to yield, feedstock quality, stress tolerance, resource use efficiency, and ecosystems services provision. Where possible examples are provided of genetic or genomic work to understand or enhance these traits.

It can be seen that, as for agriculture, advanced NGS, high-throughput genotyping, and molecular breeding techniques are helping to drive the development of dedicated, non-food biomass crops. The remainder of this article reviews recent reports of the application of these technologies to bioenergy feedstocks and includes examples of progress in relation to many of the traits discussed earlier and in Table 1.

Genotyping-by-Sequencing and Genome-Wide Association Study for Bioenergy

A **genome-wide association study (GWAS)** genotypes entire populations for **single nucleotide polymorphisms (SNPs)** and employs this extensive marker set in conjunction with

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Table 1. Target Traits for Breeding for the Sustainable Intensification of Bioenergy Poplar

Target	Trait	Description/Context	Genetics/Genomics Studies
Yield	Leaf development	Leaf size, specific leaf area, and leaf number increment have been linked to whole tree growth and are valuable for selection of high-yielding poplar hybrids [85].	Robust yield quantitative trait loci (QTL) mapped in poplar [86]. Leaf area and biomass increased in transgenic poplar overexpressing PagBEE3L, a brassinosteroid-induced transcription factor [87]. QTL mapped for leaf shape variation [88].
	Canopy duration	Canopy duration (time from bud flush to bud set) is positively associated with biomass yield by extension of the growing season [89].	Associations for canopy duration and related phenology traits identified through GWAS in *Populus trichocarpa* [31].
	Photosynthetic efficiency and stomatal patterning	Photosynthetic rate is a major target for improving food crop yields from the biochemical to the canopy level [90]. In poplar there is a positive relationship between abaxial stomatal density and biomass production [91].	GWAS identified candidate genes for stomatal patterning and link with carbon gain in *P. trichocarpa* [92]. Genetic modification to improve photosynthetic efficiency has been reported in tobacco [93].
	Cell elongation and proliferation	Increased vegetative meristem activity resulting in increased cell division or elongation should increase harvestable biomass yield in a range of second generation bioenergy crops [94].	Accelerated xylem development and enhanced biomass in transgenic poplar by overexpression of PagBEE3L, a brassinosteroid-induced transcription factor [87].
	Biomass allocation	Above- and belowground patterns of biomass allocation are under genetic control in poplar with aboveground biomass available for harvest increasing proportionally with age [95].	QTL identified for biomass allocation in poplar mapping populations [95].
	Sylleptic branching	Sylleptic branch number has a generally, but not ubiquitously, positive relationship with biomass yield in poplar [89,96].	Sylleptic branching can be induced in poplar by the transgenic expression of the DNA-binding protein CsRAV1 [97].
Feedstock Quality	Cellulose quantity and structure	The enzymatic hydrolysis of cellulose yields glucose for fermentation to bioethanol. Poplar species and hybrids have cellulose contents ranging from 42% to 49% [98]. Cellulose crystallinity is a cause of recalcitrance and reduced saccharification potential [98].	Associations have been reported from GWAS for cellulose content and cell wall crystallinity in poplar [30]. Reduced crystallinity and enhanced biochemical conversion has been reported in a transgenic cellulose synthase Arabidopsis mutant [99].
	Lignin quantity and quality	A key trait for the control of woody feedstock recalcitrance. Reduced lignin content or lignin with a high syringyl/guaiacyl (S/G) ratio is associated with higher yields of fermentable sugars [100].	GWAS has identified associations for total lignin, soluble lignin, and S lignin contents in *P. trichocarpa* [30]. Low-lignin poplar transgenics have been reported with improved saccharification potential and sugar yield [101].
	Wood density	Density is an important trait for both biomass yield and quality and shows genotypic variation between poplar hybrids [102].	Transgenic poplar expressing pine glutamine synthetase (GS1a) has been shown to possess a range of enhanced wood quality and chemical traits including wood density [45].

Table 1. (continued)

Target	Trait	Description/Context	Genetics/Genomics Studies
Stress Tolerance	Pest/disease resistance	Pests and disease can have highly negative implications for yield. The most costly poplar pathogen is the rust fungus (*Melampsora*) and resistance is a major commercial breeding priority [103].	QTL mapping for resistance QTL [103]. Associations for rust (*Melampsora* × *columbiana*) resistance identified through GWAS in *P. trichocarpa* [32].
	Drought tolerance	An important trait in the face of global climate change. Poplar is vulnerable to drought-induced cavitation [104] but the more tolerant *Populus euphratica* has been shown to respond plastically to water table depth by adjusting both root length and total root biomass allocation [105].	Transgenic poplar expressing the Arabidopsis transcription factor EDT1/HDG11 show improved drought tolerance [106]. Transcriptome resequencing from control and drought-stressed leaves of *P. euphratica* identified gene candidates related to stress perception and signalling, transcriptional regulation, and stomatal closure inhibition [107].
	Salinity tolerance	High soil salinity resulting from naturally saline groundwater or excess irrigation can cause osmotic stress to which poplar is vulnerable [108].	The salt-responsive transcriptome of poplar has been mapped [108]. The overexpression of a manganese superoxide dismutase can increase salt tolerance in transgenic poplar [51].
	Flood tolerance	Flooding and root hypoxia can cause reductions in stomatal conductance and photosynthetic rate; however, poplar clones have been shown to differ in their response [109].	No published literature on the genetic basis of flooding in poplar; however, QTL mapping for waterlogging tolerance and the identification of candidate genes associated with anaerobic responses has been performed in maize [110].
Resource Use Efficiency	Water use efficiency	A high priority to reduce vulnerability to drought and the need for supplementary irrigation in these predominately riparian species.	QTL mapped for stomatal conductance and leaf carbon isotope discrimination, an indicator of leaf water use efficiency [111]. The ectopic expression of pine glutamine synthetase has been shown to cause enhanced tolerance to water stress through the maintenance of photosynthetic electron transport capacity [47].
	Nitrogen use efficiency (NUE)	NUE important for minimising the need for fertiliser application to bioenergy poplar. Poplar nitrogen nutrition has been extensively reviewed [112].	QTL mapped for growth and wood chemistry in response to varying nitrogen availability [113]. Transgenic expression of glutamine synthetase shown to improve NUE [46].
Ecosystems Services	GHG balance	There is considerable interest in the net balance of GHG emissions (CO_2, CH_4, and N_2O) from short rotation coppice (SRC) poplar plantations after conversion from agriculture or grassland [114].	This trait represents an amalgamation of many different factors including photosynthetic efficiency, carbon assimilation, and soil carbon sequestration, as well as the need for fertilisation, which is a product of crop NUE [114].
	Volatile/isoprene emissions	Isoprene (and other organic volatile) emissions contribute to the formation of photochemical smog and reduced air quality. Poplar genotypes vary in their	RNA interference has been employed in transgenic poplar to reduce isoprene emissions without impacting biomass yield [116].

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Table 1. (continued)

Target	Trait	Description/Context	Genetics/Genomics Studies
		isoprene emissions, which do not appear to depend on photosynthetic rate [115].	
	Phytoremediation (heavy metal tolerance)	Useful for the employment of bioenergy poplar for reclaiming toxic/degraded lands.	Transgenic poplar expressing yeast cadmium factor 1 show increased tolerance to Cd, Zn, and Pb [50].
	Microorganism associations	Inoculation of poplar roots with native endophytes increased biomass yield through increased biological nitrogen fixation in a long-term field setting [117].	There are no reports of breeding attempts for fungal symbiosis specifically; however, a transgenic poplar (suppressed cinnamyl alcohol dehydrogenase) was confirmed not to impact fungal soil communities [118].

phenotyping data to elucidate the genetic basis of quantitative traits of interest [20]. NGS technologies have driven a reduction in sequencing costs and can provide genome-wide SNP discovery through complexity reducing **genotyping-by-sequencing (GBS)** approaches, which can be more cost-effective than 'gold standard' whole-genome resequencing. These methods include RNA-seq transcriptome resequencing, targeted sequence capture, and restriction enzyme (RE)-based genome fragmentation and sequencing [21]. Owing to its establishment as a model tree species [22] and the publication of the *Populus trichocarpa* (black cottonwood) genome sequence [23], studies in poplar tend to outnumber those in other candidate bioenergy species (such as willow and energy grasses) and it has been the subject of significant resequencing and genotyping efforts. These have included whole-genome resequencing [24] and both sequence capture and transcriptome resequencing [25] GBS approaches for SNP discovery. Transcriptome resequencing has also been reported in willow [26], switchgrass [27], and *Miscanthus* [28]; in the case of *Miscanthus* permitting the construction of a genetic linkage map for this species, which will be valuable for marker assisted breeding for bioenergy.

The GBS and discovery of more than 500 000 SNPs [24,25] in *P. trichocarpa* led to the development of a 34 000 SNP genotyping array for this species covering more than 3500 genes [29] and this has permitted a number of GWAS within the past 2 years. These have been supported by the finding from whole-genome resequencing that linkage disequilibrium (LD) decay in poplar is extensive enough to make an association genetics approach feasible in this species with less markers than previously anticipated [24]. Two recent papers [30,31] have reported hundreds of trait–marker associations for many of the commercially important traits that were considered earlier as bioenergy breeding priorities. These include wood chemistry traits such as lignin content and composition (a key target for improving the efficiency of feedstock processing and conversion to biofuel), biomass yield, and water use efficiency. This array was also employed to identify markers in 26 genes associated with rust severity [32], a major source of fungal infection in commercial poplar plantations and responsible for reduced biomass yields and commercial losses. In an exciting development for *Miscanthus*, more than 100 000 SNPs were recently used in a GWAS to identify trait–marker associations for phenology, cell wall composition, and biomass traits [33]. Table S1 (in the supplemental information online) summarises the key literature on the progress of advanced genotyping and association genetics in four promising cellulosic feedstocks for bioenergy. Adoption of poplar as a model tree species more than a decade ago [22] and the subsequent effort employed in its sequencing [23] has enabled progress both in numbers of SNPs called and in trait–marker associations identified.

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By comparison, *Miscanthus* has begun to make progress more recently with the establishment of association mapping populations and the publication of an extensive GBS and GWAS report last year [33]. Switchgrass has also been extensively resequenced and genotyped but has not yet been subject to GWAS. Willow is the least advanced of the four feedstocks without published GBS or GWAS at this time.

Trait–marker associations arising from GWAS can be employed for MAS, which utilises them to identify high-value individuals within a population. This permits selection for breeding at an earlier developmental stage than previously possible and improves selection time and efficiency [4]. The calling of increasingly large marker sets means that whole-genome MAS approaches are now becoming feasible [34], while increasing marker density may soon permit GS within plant species [35], already widely utilised in animal breeding. GS sees phenotyping in a large training population followed by assignment of breeding values for individuals based solely upon genotyped, trait-associated markers [1]. Modelling studies suggest that GS in forest trees could result in large increases in selection efficiency and permit significant reductions in the breeding cycle [36]. This is an excellent prospect for bioenergy poplar and willow where traditional breeding may take decades as well as requiring a large amount of space to maintain a reproductively mature population. *Miscanthus* also possesses significant phenotypic and genetic variation, suggesting that immediate implementation of GS may now be feasible in *Miscanthus* breeding programmes following on from their successful GBS and GWAS [33].

Genetic Modification for Bioenergy

An alternative route to trait improvement is provided by GM; the benefits and controversies of which have been much publicised in relation to food crops (Box 1) and the potential of which for bioenergy has been reviewed for both grasses [37] and woody crops [38]. Lignin recalcitrance is a major technical barrier to the realisation of cost-effective and sustainable second generation feedstocks, creating a requirement for energy intense and potentially costly thermochemical pretreatments before polysaccharide saccharification to yield simple sugars for fermentation [39]. Thus, understanding the genetic basis of lignin biosynthesis and the control of lignin content and composition have been significant research priorities. Much effort has been invested in low-lignin transgenics, developing knockouts or employing RNA silencing both in the model organism Arabidopsis (*Arabidopsis thaliana*) and in bioenergy crops themselves [40]. The cell wall has been extensively targeted in transgenic poplar through downregulated or altered lignin biosynthesis. The approach has been successful in reducing recalcitrance and increasing ethanol yield and conversion efficiency but often with inferior fitness and yield penalties in the field (Box 2). There have also been concerns that overlap between the lignin biosynthetic and plant defence pathways could render low-lignin transgenics vulnerable to pests and infection, especially to rust

Box 1. Policy and Public Acceptance Key to Genetic Modification Bioenergy Deployment

GM crops for human consumption are now widely deployed globally, but have also been subject to significant controversy and opposition. First approved in 1997 'MON 810', a Bt toxin expressing maize, is the only GM crop currently cultivated commercially within the EU. A second GM crop (the 'Ampflora' potato) was not approved until 2010 after a 14-year process and was withdrawn from the market just 2 years later. Gaining approval for a new GM crop within the EU is subject to Regulation (EC) 1829/2003, which requires rigorous risk assessment and risk management [119]. Even post-approval member states continue to exercise the power to refuse to permit the sale of a given GM product within their borders in response to public concern, with Directive (EU) 2015/412 to increase policy flexibility for individual nations coming into force in April 2015. Unapproved GM organisms (GMOs) are subject to zero tolerance thresholds despite the economic risks this policy poses [120]. In the USA, GMOs have been in the human food chain since 1996 but are not without controversy, with many companies responding to consumer pressure by becoming 'GMO-free' [121]. It follows that whether GM bioenergy receives investment (public or private) and is subsequently commercially deployed will depend as much on political as scientific progress and not only on successful outcomes in field trials. Transformation protocols are well established for poplar [122] and are now emerging in willow [123], *Miscanthus* [124], and switchgrass [125]. It is possible that as non-food crops and with clear environmental benefits, these GM bioenergy crops will be met with less opposition; however, early public engagement is essential.

Box 2. Lignin Reduction is a Two-Edged Sword

Inferior fitness and biomass yield reductions have been associated with a number of trials of poplar low-lignin transgenics in recent years. The enzyme cinnamoyl CoA reductase (CCR) is responsible for the penultimate step of monolignol synthesis. Two field trials of CCR-deficient poplar reported improved pulping characteristics [126] and enhanced ethanol yield [101], respectively, but accompanied by yield reductions of greater than 30% and 16% to 24%. Similar yield penalties accompanying lignin reduction were also found for the downregulation of 4-coumarate:coenzyme A ligase (4CL) [127], as well as reduced wood strength and stiffness [128]. More minor reductions in tensile strength and stiffness were reported when cinnamate 4-hydroxylase (C4H) was downregulated resulting in a 30% decrease in lignin content [129]. It thus appears that there are limits to the gains that can be safely secured from blunt reductions in lignin content as yield penalties can outweigh feedstock quality improvements. Low-lignin transgenics must be subjected to rigorous, long-term field testing before commercial deployment to ensure reliable yields in the face of biotic and abiotic stressors and may be of limited value for sustainable intensification. Such stresses are likely to become more prevalent in the face of a changing climate as a result of drought, extreme weather, and the emergence of new or alternately distributed pathogens.

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[41], which is a major poplar pathogen, although as yet there is no evidence to support this contention. The defence of poplar against rust infection is based on the increased expression of genes within the phenylpropanoid pathway and the accumulation of monolignols [42]; it follows that genetic modifications targeting this pathway could unintentionally increase rust susceptibility in bioenergy poplar plantations [41]. A highly novel and promising approach has sought to overcome these issues by modifying the structure rather than reducing the content of lignin [43]. This was achieved by the transgenic expression of the enzyme monolignol ferulate transferase, which inserts ester linkages into the backbone of the polymer, creating a lignin amenable to depolymerisation by a far milder pretreatment protocol but with the transgenic poplar showing no reduction in lignin content or any phenotypic abnormality in the glasshouse environment. By reducing the severity of the necessary pretreatment, it is hoped that this approach can greatly improve the cost-effectiveness and sustainability of lignocellulosic bioethanol. Beyond poplar, targeting of the lignin biosynthetic pathway has also succeeded in improving ethanol yields in transgenic switchgrass. Promising results from a 2-year field trial of transgenic, low-lignin switchgrass showed increased sugar release and ethanol yield without a biomass yield penalty or increased rust susceptibility in this energy grass species [44].

Outside of the lignin biosynthetic pathway, the ectopic expression of pine glutamine synthetase (*GS1a*) in poplar has been shown to impart wide improvements in wood chemistry and increased lignin solubility without negatively impacting yield [45]. Transgenic lines showed increased S-lignin content as well as elevated concentrations of the wood sugars, glucose, galactose, mannose, and xylose, suggesting that *GS1a* overexpression may be a promising route for biofuel development. *GS1a* is a key component in nitrogen metabolism and has also been linked with increased nitrogen assimilation efficiency leading to enhanced growth [46], as well as improved drought tolerance [47]. In the latter instance, this tolerance appeared to be mediated through increased expression of photosynthetic enzymes and higher chlorophyll content under drought in the transgenic lines, helping to maintain electron transport capacity when stomatal conductance was reduced under water stress. Enhanced growth has also been reported in transgenic poplar expressing Arabidopsis nucleoside diphosphate kinase 2 (*NDPK2*), a regulator of antioxidant gene expression whose effect may be mediated through enhanced oxidative stress tolerance [48]. Growth has also been targeted in transgenic switchgrass by the overexpression of an apical dominance regulatory miRNA [49]. With a desire for second generation biofuels to be cultivated on marginal or degraded lands to avoid competition with agriculture, consideration has been given to the potential for the use of transgenic feedstocks for land reclamation and phytoremediation. Bioenergy plantations of this nature could be of significant local environmental benefit as well as providing low-carbon energy. Transgenic poplar trees expressing yeast cadmium factor 1 (*ScYCF1*) have shown improved growth and heavy metal accumulation on mine tailing soil [50]. Salinity is another major issue impacting soil quality and agriculture and may become increasingly significant as coastal flooding events are

predicted to increase as a result of global climate change. The transgenic increase of superoxide dismutase (SOD) activity has been shown to improve salt tolerance in poplar, leading to greatly improved growth in saline soil [51]. The successful deployment of CRISPR/Cas (clustered regularly interspaced short palindromic repeat/CRISPR-associated) technology in poplar this year is an exciting development that may open up further possibilities for precision **genome editing** in this, and other, bioenergy crops [52].

Concluding Remarks

We have provided a brief overview of the progress that has been made in the application of NGS, advanced genotyping, association genetics, and GM in lignocellulosic bioenergy crops, most widely deployed at present in poplar. These molecular techniques will underpin the sustainable intensification of new non-food plants that may in future be grown over extensive tracts of marginal agricultural land. These examples have already provided promising results with higher yielding and more stress-tolerant GM lines reported and large numbers of markers/candidate genes identified for a wide array of key bioenergy traits including growth, disease tolerance, and feedstock quality. Traditional breeding programmes have yielded significant improvements in bioenergy crops, for example, the doubling of willow biomass yields in the past 30 years [53]. Now these new advances, driven by molecular genetics, will open the way to the application of marker assisted breeding and GS in second generation biofuels for further, more rapid progress. The improvements made in food crops so far show the pivotal role advanced breeding can play in ensuring the sustainable intensification of second generation biofuels (see Outstanding Questions). How significant the role will be for GM feedstocks is unclear, depending on successful outcomes from rigorous field testing as well as governmental approval and broad public acceptance, but genomic strategies for selection and breeding are now a reality and are likely to drive breeding programmes forward in the future, with or without GM deployment. We can be optimistic that the large yield gap in these non-food outbreeding, unimproved crops is a tractable target for several new DNA approaches. In conclusion, the successful pursuit of advanced breeding programmes will be central to the development of high-yielding, sustainable non-food bioenergy crops as nations around the world seek to meet their renewable energy commitments.

Acknowledgments

Supplementary Information

Supplementary information associated with this article can be found online at http://dx.doi.org/10.1016/j.tplants.2015.10.002.

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Outstanding Questions

Will less developed lignocellulosics receive the attention and funding needed to achieve their wide-scale deployment? The model tree poplar has far exceeded willow, *Miscanthus*, and other species in NGS, genotyping, and GM to date; however, association populations and molecular approaches are beginning to be reported in these crops. A diversity of feedstocks suited to a variety of environmental conditions will be vital if second generation bioenergy is to achieve more than marginal significance in the future energy mix. Can competitive yields be achieved on marginal lands with low-input agricultural practices? Many high-yielding bioenergy trials have received supplemental irrigation and/or fertilisation. Advanced breeding for nitrogen and water use efficiencies will need to overcome a large yield gap if profitable yields are to be obtained on poor soils while minimising life cycle carbon emissions.

Can GS and genome editing be considered acceptable biotechnological routes to yield intensification? Which technologies will be targeted across these lignocellulosics? Will breeding cycles (time to flowering) hinder progress in poplar?

Can promised high biomass yields be obtained when production is moved to a larger, biorefinery setting in a multifunctional landscape? Many trials of second generation feedstocks involve small plots and it may be optimistic to linearly scale these yields to a commercial setting. Conversely, larger scale production practices may bring greater efficiency to bioenergy cultivation and conversion resulting in more favourable life cycle energy and emissions costs.

Will government policy support the development of second generation bioenergy? Key issues include the extent of funding and subsidy regimes, which will determine uptake and investment in second generation bioenergy, the allocation of land for cultivation and biorefinery construction, and the restrictions placed on the deployment of GM feedstocks. Policy must work together with industry to develop an economically viable sector.

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