Invited Expert Review

Genetic Improvement of Willow for Bioenergy and Biofuels Free Access

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Abstract

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Willows (*Salix* spp.) are a very diverse group of catkin-bearing trees and shrubs that are widely distributed across temperate regions of the globe. Some species respond well to being grown in short rotation coppice (SRC) cycles, which are much shorter than conventional forestry. Coppicing reinvigorates growth and the biomass rapidly accumulated can be used as a source of renewable carbon for bioenergy and biofuels. As SRC willows re-distribute nutrients during the perennial cycle they require only minimal nitrogen fertilizer for growth. This results in fuel chains with potentially high greenhouse gas reductions. To exploit their potential for renewable energy, willows need to be kept free of pests and diseases and yields need to be improved without significantly increasing the requirements for fertilizers and water. The biomass composition needs to be optimized for different end-uses. Yields also need to be sustainable on land less productive for food crops to reduce conflicts over land use. Advances in understanding the physiology and growth of willow, and in the identification of genes underlying key traits, are now at the stage where they can start to be used in breeding programs to help achieve these goals.

Karp A, Hanley SJ, Trybush SO, Macalpine W, Pei M, Shield I (2011) Genetic improvement of willow for bioenergy and biofuels. *J. Integr. Plant Biol*. **53**(2), 151–165.

Introduction

Throughout history, people have used willows for practical uses, healing, and spirituality. The druids considered the willow sacred and the willow tree is associated with death and rebirth in Celtic and Middle Eastern cultures. Many willows provide basket-making material and records of willow coracles date back to Herodotus in 5th century BC. In the UK, willow baskets were used in WWI as mule panniers to carry artillery shells to the front and in WWII to parachute supplies to the troops.

Cricket bats are made from white willow (*Salix alba*) and bark of this species was traditionally used to treat fevers and as an analgesic. The first record of this use dates back to Hippocrates in circa 400 BC but it was not until 1828 that the active extract of the bark (salicin) was isolated in crystalline form and it took many more years before it was marketed world-wide as aspirin.

Basket-making remains popular today and willows are also still woven to make windbreaks, hurdles and sculptures. Many willow species are also enjoyed as living sculptures, and as ornamentals in gardens, or large trees in natural and planted

landscapes. Despite these continued interests and uses, willow received only limited attention in terms of research on biology, physiology and genetics. The need for this only really emerged when renewable sources of energy were sought for heat and power generation during the oil crisis of the 1970s and when new interest in willow as a source of biomass arose. Willow was identified as a promising biomass crop due to its ease of propagation and ability for fast growth in short rotation coppice (SRC) cycles with only minimal fertilizer inputs. In SRC, small (approximately 20 cm) stem cuttings are planted in spring at high densities (10 000–20 000/ha) and after the first year's growth the stems are cut back once the leaves have dropped. In the following spring the cut stumps (stools) re-sprout to provide multiple shoots which reach approximately 5 m in height in 3 years, after which they are harvested. New shoots re-sprout and the SRC cycle is continued for around 20–25 years. The cycle is shorter than traditional forestry, which makes it more suitable for arable farmers. SRC has now been adopted in many countries, especially in northern Europe, North America, Canada, India, New Zealand and Japan (Kuzovkina et al. 2008).

Increasing concerns over climate change and energy security have heightened interest in the development of renewables, not only for heat and power but also for transport fuels, and willow has a recognized contribution to make. However, willow varieties initially used for biomass plantations were originally selected for basket-making and optimization of the crop is needed to fully realize the potential of SRC willow for bioenergy and biofuels. Here we review the advances made in our understanding of key traits for crop improvement and the potential for identifying the underlying genes for marker-assisted selection to assist genetic improvement of willow for future bioenergy and biofuel markets.

Genetic Diversity

Salix (willow), together with *Populus* (poplar, aspen, and cottonwood), constitute the family *Salicaceae* s. str. of the order *Salicales*, class *Magnoliopsida*, subclass *Dilleniidae* (Kuzovkina et al. 2008). There are about 330–500 species of *Salix,* widely distributed throughout the Northern Hemisphere, with only a few species native to the Southern Hemisphere (Argus 2007). They are mostly found in temperate and arctic zones but a few have adapted to subtropical and tropical zones. The centre of diversity is believed to be in Asia, with around 275 species in China (189 endemics). Around 120 species are found in the former Soviet Union, over 100 in North America (one species is native to South America) and around 65 species in Europe (Argus 2007).

It is hard not to be impressed by the diversity of form that can be found in the genus *Salix* (Figure 1). Willows range from the tall trees, including weeping forms, through shrubs and bushes,

to prostrate, dwarf and rockery plants (Figure 1). All willows are deciduous with simple leaves in an alternate or occasionally opposite arrangement. However, leaf size varies enormously (Figure 1A–F), for example, from the long slender leaves of *S. viminalis* (Figure 1A) to the large broader leaves of *S. pentandra* (Figure 1D) and especially *S. magnifica* (Figure 1C). Curly forms exist (Figure 1E) and leaf surfaces may be smooth, hairy, dull, or lustrous, or occasionally covered on the lower side with a whitish waxy bloom. Stomata are dense on the lower leaf surface but are found on both surfaces in some species. Stems show a wide variety of colors (e.g. *S. purpurea*; Figure 1G) and include twisted and fasciated forms.

Willows are dioecious, with male and female trees bearing distinct catkins (Figure 2). They have separate flower and vegetative buds but a characteristic of willows is that all buds are covered with a single scale. The flowers are insect- or wind-pollinated (Argus 1974) and are mostly borne on erect catkins, which appear before or at the same time as the leaves. Willow seeds are shed 3–8 weeks after pollination and are wind-dispersed. The seeds are tiny (0.8 to 3.0 mm) and, because they lack endosperm, must germinate immediately. However, sufficient moisture must be present, which may explain why willows are often found in riparian habitats.

Classification of the genus *Salix* is notoriously difficult (Linnaeus 1753) and, even today, identification at the species level remains problematic for a variety of reasons. Many species show phenotypic plasticity and there is considerable inter-specific hybridization and variation in ploidy. Non-concurrent phenology for flowers and leaves in some species also means that morphological characters cannot be seen at any one specific time. The several authoritative treatments that exist are being continuously revised but among the most widely adopted are those of Argus (Argus 1997) and Skvortsov (Skvortsov 1968), which divide the genus *Salix* into four subgenera: *Salix*, *Longifoliae* Andersson, *Vetrix* Dum, and *Chamaetia* Nasarov (Kuzovkina et al. 2008).

Molecular techniques have been used to help address problems in willow phylogeny and to characterize diversity among biomass species and clones. Phylogenetic studies include determination of relationships among species in the section *Longifoliae* using chloroplast DNA (cpDNA) (Brunsfeld et al. 1992). However, insufficient variation was found in a ribosomal DNA region comprising 5.8S RNA and the internal transcribed spacers ITS 1 and ITS 2 to fully resolve relationships among 13 species in the *Salicaceae* (Leskinen and Alstrom-Rapaport 1999). Similar low levels of diversity were found in the large subunit of ribulose-1,5-bis-phosphate carboxylase/oxigenase (*rbcL*) among 19 species of the genus *Salix* (Azuma et al. 2000).

Early approaches to characterize biomass clones used fingerprinting methods such as randomly amplified polymorphic DNAs (RAPDs) and M13 universal primers, as well as isozymes
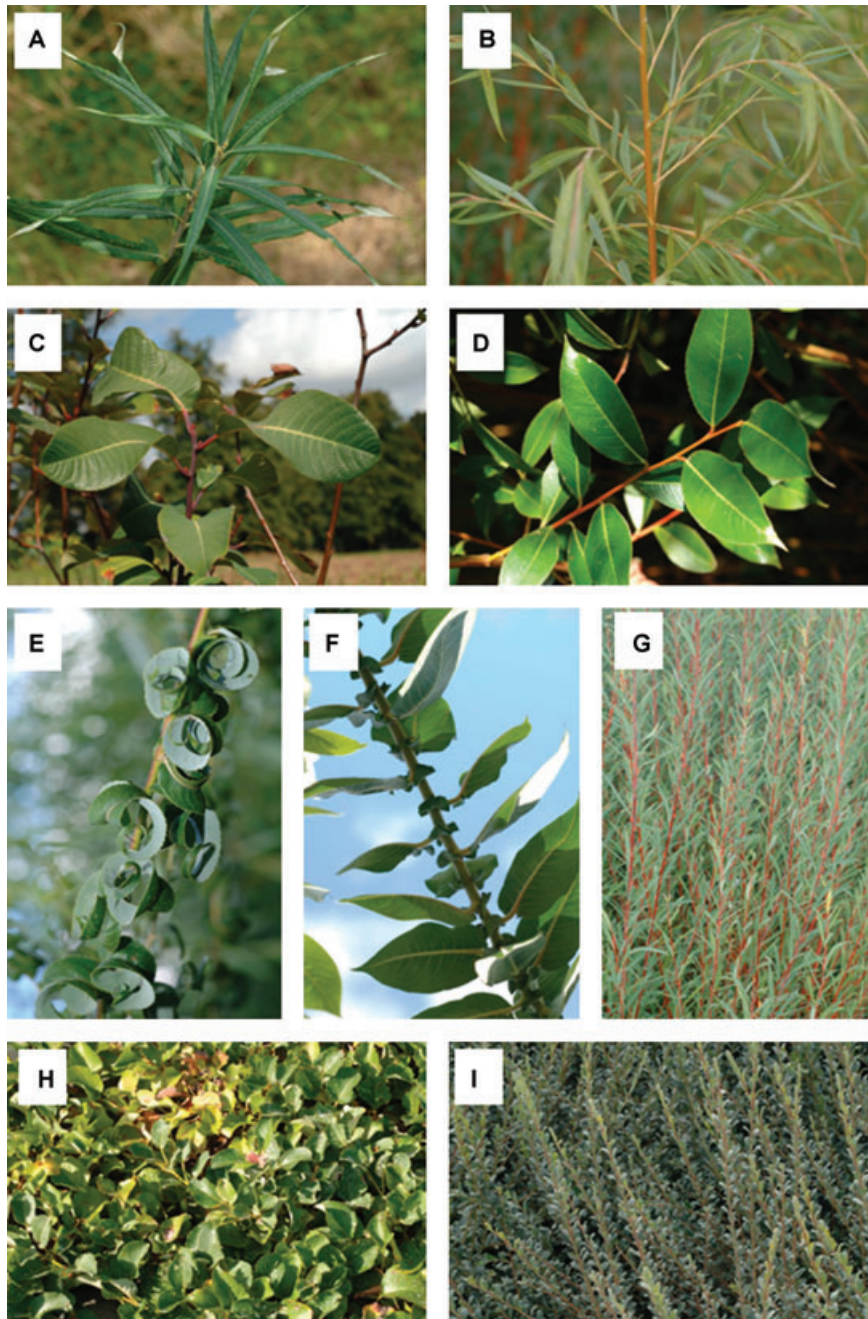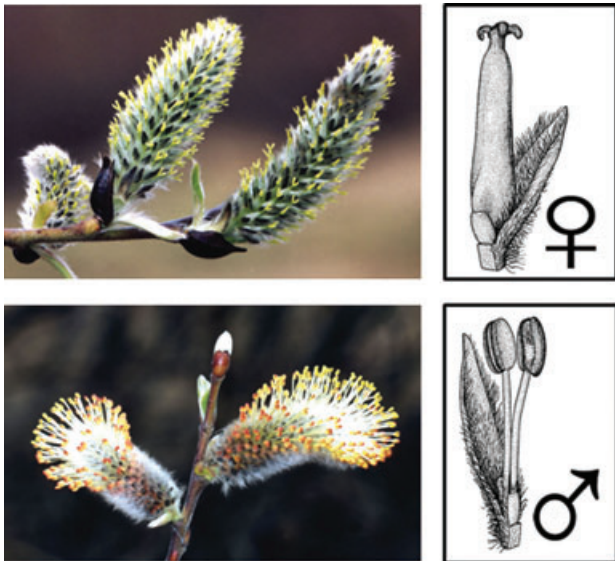
Figure 1. Examples of diversity in leaf (A–F), stem (G) and growth (H-I) form in willow (*Salix* spp.).

(A) *S. viminalis*.

(B) *S. alba* var.*vitellina pendula* × *S. babylonica*.

(C) *S. magnifica* Hemsl.

(D) *S. pentandra* L. var. Dark French.

(E) *S. babylonica* L. var. Annularis.

(F) *S. cordata* Muhl.

(G) *S. purpurea* L.

(H) *S. nakamurana*

(I) *S. repens*.

Figure 2. Examples of dioecious flowers in willow: (upper) female catkin; (lower) male catkin.

(e.g. Chong et al. 1995; Thorsen et al. 1997; Aravanopoulos et al. 1999). Microsatellites (Hanley et al. 2002) and amplified fragment length polymorphism (AFLP) are more discriminative and reproducible and have since proven very useful, for example, in distinguishing between natural populations of *S. alba*, *S. fragilis* and their hybrid *S*. × *rubens* (Beismann et al. 1997; King et al. 2010) and between a set of 29 biomass cultivars (Barker et al. 1999). Although microsatellites have the advantage of being co-dominant markers, AFLPs may be more useful in diversity studies aimed at covering a wide range of willow species and interspecific hybrid clones, where polyploidy and problems with cross-amplification may limit the application of microsatellites. Genetic relationships among 154 willows, including 50 species, were successfully resolved using nine primer combinations in an optimized fluorescent AFLP protocol (Trybush et al. 2006, 2008). The study was based on willows from the UK National Willow Collection and included 40 species used in breeding. The results challenged all current classifications, which assign *S. triandra* to subgenus *Salix* and have since been supported by results from the chloroplast rbcL gene, trnD-T spacer and atpB-rbcL spacer regions (Chen et al. 2010).

Breeding and Genetics

For most commercial forest tree species, generation turn-over periods are too long to make multiple generation breeding possible. For willow, however, most species will flower by the second year of growth from a seed, or a cutting, and often within the first year. Considering also that wide genetic diversity exists in the germplasm, that emasculation is not required for crossing, that willows hybridize readily (at least within sub-genera), that seed set can be very high, and that progeny can be easily multiplied as stem cuttings, willow has many advantages for both breeding and genetics compared with many other tree species. However, there are also challenges to be faced. Although many species are diploid ($2n = 2x = 38$) ploidy levels can reach up to dodecaploid ($2n = 12x = 228$) and many New World and European species, as well as many biomass varieties, are polyploid (Macalpine et al. 2008). Willows are highly heterozygous and cannot be selfed to form inbred lines. Crossing barriers do exist among some species (Macalpine et al. 2008) and dioecy can be a problem if genotypes desired for crossing are of the same sex. There are few traits that can be scored reliably in the nursery and although there are non-destructive methods of estimating yield, based on stem heights and diameters, true (harvested) biomass yield can only be assessed after 4 years and then in 3 year cycles. Measuring traits is not trivial in a mature coppice plantation and trials are expensive to maintain on multiple sites and to assess for long periods of SRC cycles.

Despite these difficulties, willow breeding advanced after the 1970s through conventional selection in the hands of a few pioneering individuals particularly in the UK, Sweden, New Zealand and the US (Kuzovkina et al. 2008). In the UK, willow breeding started at Long Ashton Research Station (LARS) in Somerset, where research can be traced back to 1922 when the UK government appointed a first willows office (Hutchinson) to support the use of willow baskets for the war effort. Hutchinson collected different basket-making varieties and began the National Willow Collection, which now holds over 1 300 genotypes, including circa 100 species and is a valuable germplasm repository for breeding (**Figure 3A**). When willow became recognized as a promising biomass crop, Stott (1984) characterized 54 species from the Collection and identified shrub willows (such as *S. viminalis*) as being most suitable for biomass. Stott initiated a crossing program at LARS, which later led to the release of varieties Ashton Stott (or Stott 10) and Ashton Parfitt. In Sweden, Larsson (1998) similarly described the importance of shrubby species such as *S. viminalis* and *S. dasyclados* as well as *S. schwerinii*, *S. triandra*, *S. caprea*, *S. daphnoides* and *S. eriocephala*. The Swedish breeding program, initiated by Svalöf Weibull AB (now Lantmännen) in 1987 developed many improved varieties such, as Tora, Björn and Torhild, which are widely grown today. In 1996, the UK and Sweden combined their efforts to form the European Willow Breeding Partnership (EWBP) and seven EWBP varieties were released (**Table 1**).

The EWBP was dissolved and breeding now continues separately in Sweden and in the UK, after LARS closed and the entire willow program was relocated to the main Institute site at Rothamsted Research in Hertfordshire in 2002. The
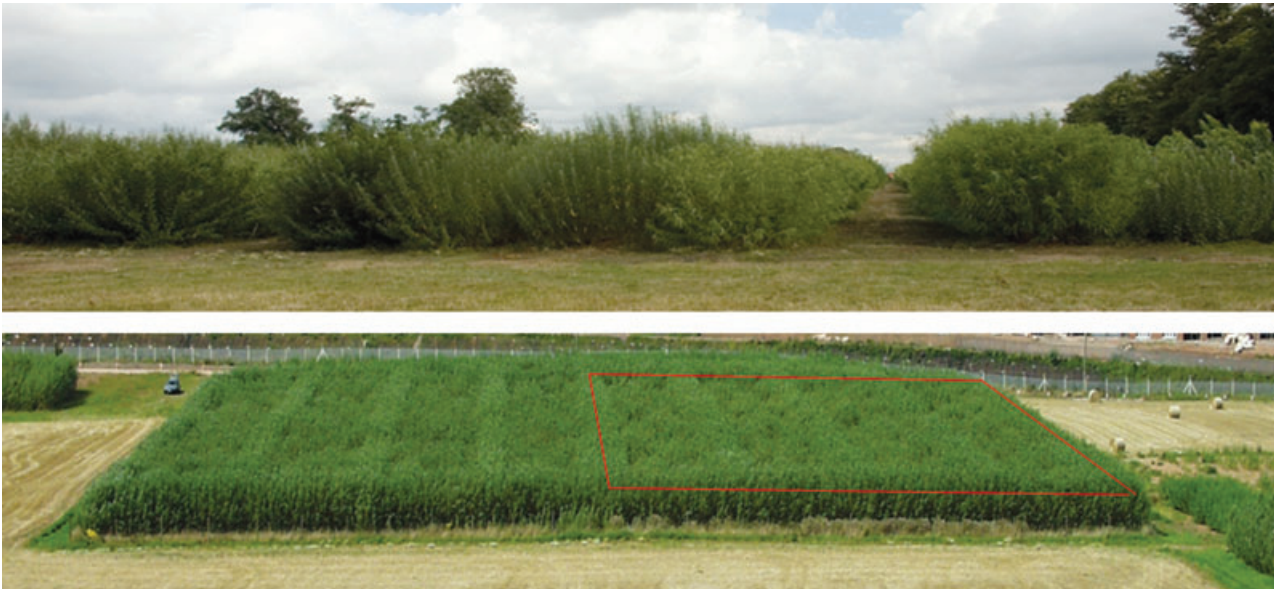
Figure 3. Genetic resources in willow: (upper) National Willow Collection at Rothamsted Research; (lower) Mapping populations growing at the Long Ashton site (K8 is delineated by the red box).

current program at Rothamsted performs between 50 and 150 crosses and generates some 7 000–12 000 seedlings each year. Since 2002, 1 432 lines have been advanced from the nursery to first un-replicated (Observation 1) trials and 48 breeding lines to second (Observation 2) trials, replicated at two sites. Seven elite lines have been identified to date and are currently in breeding trials with commercial growers. Many contain *S. viminalis,* which has also been popular in the breeding programs in Canada, in addition to *S. eriocephala*. In the US, breeding has mostly concentrated on *S. eriocephala*, *S. miyabeana* and *S. purpurea* (Kuzovkina et al. 2008).

Throughout the above period, biomass yields of willow have doubled from < 7 to circa 14 ODT/ha per year (**Figure 4**). These increases were achieved at a time when there was little under-

standing of the genetics of many important traits. Most selection has been carried out directly on the hybrids created from intra- and inter-specific crosses, although other strategies such as the use of recurrent selection have been discussed (Gullberg 1993). Selections were made on the basis of observed stem characteristics (height, diameter, straightness) and coppicing response (number of shoots, shoot vigor) as well as resistance to pests, diseases and environmental stress (Larsson 1998).

Around the same time, however, the foundations were also being laid for genetic studies and for the identification of quantitative trait loci (QTL). A number of crosses were established, of which the largest (K8; $n = 967$) was created in 1999 at LARS (**Figure 3B**). K8 was subsequently duplicated at Rothamsted and has now also been planted on a third contrasting site. An

Table 1. The seven cultivars released from the European Willow Breeding Partnership (EWBP)

Cultivar	Release date	Breeders code	Female parent	Male parent
Beagle	2003	LA960326	*S. viminalis* (Astrid)	Polycross, but possibly *S. viminalis* (Orm)
Quest	2003	LA960231	*S. viminalis* (033/08, Pavainen E7899)	*S. schwer.* × *vim.* (Bjorn)
Discovery	2006	LA980024	*S. schwerinii* (109/03)	*S. schwer.* × *vim.* (Bjorn)
Resolution	2006	LA980414	*S. vim.* × *vim.* × *S. schwer.* × *vim.* SW930812 (Jorunn × Bjorn)	*S. vim.* × *S. schwer.* × *vim.* (Quest)
Endeavour	2007	LA970164	*S. schwerinii* (109/03)	*S. viminalis* (Jorr)
Nimrod	2007	LA980125	*S. schwerinii* × *viminalis* L. (Tora)	*S. miyabeana* L. (239/01, Shrubby willow ex. China)
Terra Nova	2007	LA980132	*S. triandra* L. × *viminalis* L. (*S. mollissima*, LA940140)	*S. miyabeana* L. (239/01, Shrubby willow ex. China)

$$y = 0.1x - 189.92$$
$$R^2 = 0.264\,7$$
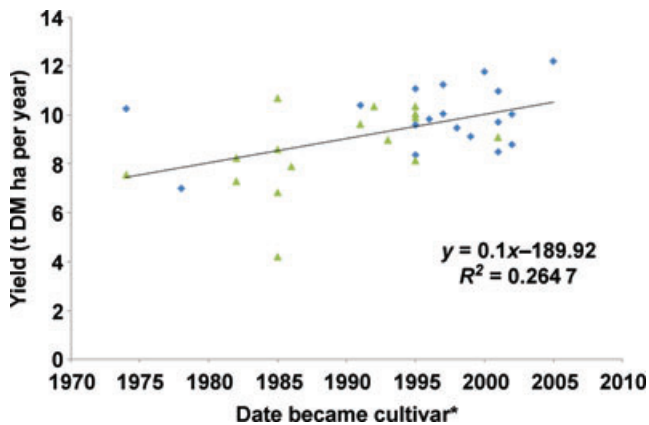
Figure 4. Increase in biomass yields of willow between 1975 and 2005.

Yields are expressed as averages for successive years from published site-trial data (*Date of application to Community Plant Variety Office (CPVO) post-1995 or first appearance in trials pre-1995; ♦ data from Lindegaard et al. 2001; ▲ data from Kightley et al. 2008. DM, dry matter.

additional 11 populations have since been made at Rothamsted for mapping purposes, most of which have in the order of 500 progeny. Several smaller *S. viminalis* crosses have also been generated in Sweden, and in the US, mapping families have been established for *S. eriocephala* and *S. purpurea*.

Using these resources QTLs have been mapped for a number of traits including biomass growth, i.e. shoot height, diameter, and number (Tsarouhas et al. 2002; Hanley 2003), frost tolerance and phenology (Tsarouhas et al. 2003, 2004) water-use efficiency, drought tolerance (Rönnberg-Wastljung et al. 2005; Weih et al. 2006) rust resistance (Hanley 2003; Tsarouhas et al. 2003), insect resistance (Rönnberg-Wastljung et al. 2006) and saccharification potential (Brereton et al. 2010). More recently, in an EraNet funded project, the Swedish and UK groups have again joined efforts to collect *S. viminalis* individuals from natural sources to form a population for asso-

ciation genetics. The population comprises approximately 380 plants and is planted at four contrasting sites. The aim is to identify genes that affect phenology, stress tolerance and yield on marginal land.

Poplar has received much attention as a model tree system and considerable genetic and genomic resources have become available in recent years (Yang et al. 2009), including a whole genome sequence of *P. trichocarpa* (Tuskan et al. 2006). These advances are highly beneficial for research studies in willow. Transfer of information from poplar has been facilitated by direct alignment of the K8 map to the poplar genome (Hanley et al. 2006). To achieve this, a set of genome-wide, expressed poplar sequences was selected and used to design primer sets that efficiently amplified homeologous regions in willow. Single nucleotide polymorphisms (SNPs) were used to map the loci and align the willow linkage groups to the poplar genome sequence. A high degree of macrosynteny was revealed (Hanley et al. 2006), which has proven useful for identifying candidates underlying OTL conferring traits of interest in willow.

Now that the foundations have been laid for genetic and genomic studies in willow, the stage is set for improving breeding efficiency by combining a genetic approach with studies on the physiology and fundamental biology of the plant. In particular, to exploit their potential for renewable energy, willows need to be kept free of pests and diseases; yields need to be improved without significantly increasing the requirements for fertilizers and water and biomass composition needs to be optimized for different end-uses (Karp and Shield 2008).

Diseases and Pests

Rust caused by *Melampsora* spp, (**Figure 5**) is by far the most serious disease of willows. Rust can reduce biomass yields by 40% and also predispose the affected plants to secondary pathogens, which can lead to death of the plant (Pei et al. 2005). There are several species of *Melampsora* that occur on willow but the most widespread and damaging is *M. larici-epitea* which alternates on larch (*Larix* spp.). *Melampsora larici-epitea* has

Figure 5. Short rotation coppice (SRC) willow: (left) Typical plantation after leaf drop. Three-year-old stems on one year stools, growing on the Rothamsted site. (Right) Close-up of stools showing typical mixture of dominant and smaller suppressed stems.

five spore stages and is highly specialized in its pathogenicity to willow hosts (Pei et al. 1996, 1999b). Eight *formae speciales* (six in Europe and two in the Far East) are recognized within the species (Pei et al. 2005). In the UK, a number of pathotypes (which show the same virulence/avirulence pattern on a set of host differentials) were identified under f. spp. *larici-epitea typica* (LET), *larici-retusae* (LR) and *larici-daphnoides* (LD) (Pei et al. 1996, 1999a, 1999b, 2003). AFLPs were able to differentiate among five pathotypes examined (LET1, LET3, LET4, LR1 and LD1) and place them into separate groups (Pei and Ruiz 2000).

Breeding improved willows for rust resistance requires knowledge of the genetic basis of the resistance and the identification of useful sources of resistance in the germplasm. *Salix viminalis* can be highly susceptible to rust (Pei et al. 1999b, 2004). Early studies of the inheritance of rust resistance in intraspecific hybrids of *S. viminalis*, (Gullberg and Ryttman 1993) and intra- and inter-specific F_1 hybrids of *S. viminalis* and *S. dasyclados* (syn. *S. burjatica*) revealed additive variation. In later work, seven F_1 crosses of willows were tested for rust resistance through leaf disc inoculation experiments and field disease assessments (Pei et al. 2001). The F_1 hybrids were equally or more susceptible compared with the parents in the crosses *S. disperma* × *S. burjatica*, *S. viminalis* × *S. cinerea* ssp. *oleifolia*, *S. viminalis* × *S. triandra* and *S. viminalis* 'Bowles Hybrid' × *S. viminalis* 'French Osier'. In contrast, the F_1 hybrids were more resistant than the parents in *S. viminalis* × *S. burjatica*, *S. viminalis* × *S. candida* and *S. viminalis* × *S. miyabeana* (formerly *linearistipularis)* (Pei et al. 2001).

Fortunately, far Eastern *Vimen* species such as *S. schwerinii* and *S. sachalinensis* (Syn. *S. udensis*) appear to be highly resistant to *Melampsora* rusts in Europe (Pei et al. 1996, 2004). Resistance from *S. schwerinii* has already been successfully exploited in breeding, as is evident from the rust-free status of Swedish varieties 'Tora' and 'Bjorn', which are both F_1 hybrids of *S. schwerinii* × *S. viminalis* and have been free of rust for the past two decades. Rust resistance from *S. sachalinensis* has still to be exploited in breeding programs. To investigate the potential of this species the inheritance of rust resistance was studied in two F_1, two F_2 and two backcross (BC) full-sib families of *S. sachalinensis* × *S. viminalis* using two isolates (VM and ST) of the LET1 and LET 5 pathotypes, respectively, in leaf disc inoculation experiments (Bayon et al. 2009). Ratios of resistant to susceptible plants in the progeny suggested that two independently segregating dominant genes controlled resistance against the VM isolate, and that a single dominant gene was responsible for resistance to ST (Bayon et al. 2009).

Resistant phenotypes can be identified in the field within a breeding program. However, the existence of multiple pathotypes of the rust, which may vary in intensity in different years, means that the nature of the resistance and, particularly, which genes/combination of genes are acting to confer the resistant phenotypes, is not easy to determine without the use of markers. Rust resistances have successively been overcome by the pathogen in both poplar and willow (McCracken and Dawson 1998; Pei et al. 2005). For the long term success of willow as a biomass crop it will be essential to identify a diverse complement of genes conferring resistance against pathotypes, which can be combined in different ways.

The genetic mapping of resistance loci for *Melampsora* rusts in *Populus* has been the focus of several studies (Cervera et al. 1996; Newcombe et al. 1996; Villar et al. 1996; Lefèvre et al. 1998; Stirling et al. 2001; Zhang et al. 2001; Jorge et al. 2005) but only a few attempts have been made in *Salix*. The presence of QTL for rust resistance was identified in an early study of a small *S. viminalis* × *S. viminalis* population (Hanley 2003). In later work, data from leaf disc inoculation tests, based on three rust isolates, were used to identify QTL for resistance components in two inter-specific willow mapping populations. Between 8 and 26% of the variation in rust resistance could be attributable to the QTLs in nine genomic regions in the (*S. schwerinii* × *S. viminalis*) × *S. viminalis* family and to the QTLs in seven genomic regions in the F_2 family of *S. viminalis* × *S. dasyclados* (Rönnberg-Wastljung et al. 2008). However, in both studies, progeny sizes limited the resolution with which loci could be mapped and the anonymity of the markers prevented the comparison of the QTL positions detected in the two studies.

More recently, rust resistance has been mapped in K8 (Hanley et al. In submission). In addition to offering the possibility to map with more precise resolution (due to the larger population size), the pedigree of K8 is of great interest as it includes *S. schwerinii* 'L79069', the major source of rust resistance in several modern biomass willow varieties (Larsson 2001). Rust resistance was assessed in the field and in laboratory inoculation tests using isolates of LET1 and LET5. For field-based resistance a major QTL was detected in addition to several of more modest effects. The major locus was supported by the inoculation test data. As the K8 linkage map is anchored to the publicly-available *P. trichocarpa* genome sequence (Hanley et al. 2006), comparative analysis is possible, providing an efficient route to the possible identification of the resistance gene involved.

Willows are propagated by cuttings and plantations containing a single genotype are genetically homogenous. It is known that large scale monoculture plantings are vulnerable to pathogen attacks. A strategy to reduce such a risk is to plant host genotype mixtures to increase genetic diversity. The main effect of host mixtures on disease is physical separation of susceptible host genotypes in the plantation. Another effect of mixtures is that the presence of avirulent pathotypes induces resistance in hosts, resulting in less disease (Pei et al. 2003). Unlike many foods or horticultural crops, in which flavor, color, and shape may have prime importance, genotype mixtures

are readily accepted for biomass production. Studies on SRC willow mixtures in England and Northern Ireland in the past two decades demonstrated that willow genotype mixtures delay disease onset, reduce disease levels substantially and enhance yields (McCracken and Dawson 1998; Hunter et al. 2002; McCracken et al. 2005). However, mixture plantations composed of different varieties of a single species *S. viminalis* proved to be less effective in suppressing rust disease (Begley et al. 2009). It has been suggested that the current commercial practice in many parts of Europe of planting mixtures as a disease control strategy will only be effective if there is sufficient genetic diversity between the *Salix* genotypes incorporated into the mixture (Begley et al. 2009).

Progress with respect to breeding pest resistance has been slower in comparison to rust. Willows are attractive hosts to many insect pests. Of these, Chrysomelid beetles, particularly the blue and brassy willow beetles (*Phyllodecta* (previously *Phratora*) *vulgatissima* (L.) and *P. vitellinae* respectively) and the gall midge (*Rhabdophaga*) spp. continue to be the most important in Europe. Other pests include the giant willow aphid (*Tuberolachnus salignus* Gmelin), potato leafhopper (*Empoasca fabae* Harris) and sawfly larvae (*Nematus* spp.).

It has been shown that insects are either attracted to or repelled by willow leaf volatiles, and will choose to feed, or not, depending on phenolic secondary metabolites in the leaf (Karp and Peacock 2004). Willows are highly variable in secondary chemistry and this could be exploited to select willows that are less attractive to the pest. A potential problem is that although the identity of some of these chemicals is known in willow, and candidate genes can be proposed based on knowledge in poplar and other species, it would appear that the insects respond to different concentrations of the same compounds in the volatile profile/leaves and not to their absence/presence. This could make it difficult to breed for optimal chemistry unless genes with major effects can be identified. Inheritance patterns of phenolics have been studied in relation to insect herbivory in willow species and their interspecific hybrids and the latter found to have intermediate levels compared with the parental species (Hallgren et al. 2003). QTLs for resistance to herbivores have also been identified in a *S. dasyclados* × *S. viminalis* family (Rönnberg-Wastljung et al. 2006) but, unfortunately, the tetraploid nature of the cross makes further investigation difficult. At Rothamsted, specific crosses between diploid species of willow have now been set up to map insect resistance to more tightly resolved regions of the genome.

Yield and Composition

Yield is a complex trait but breeding strategies aimed at increasing yield in willow need to take into account two additional layers of complexity due to the fact that willows are not only perennial but are also grown in SRC cycles (Karp and Shield 2008).

In SRC, willow is characterized by vigorous re-growth of multiple stems after coppicing and rapid development of a canopy, followed by self thinning of suppressed stems (Sennerby-Forsse 1995; Ross and Ross 1998; Sannervik et al. 2006) (**Figure 5**). Coppicing releases dormant axillary buds in the stool from apical dominance, allowing new shoot development to take place, and also re-invigorates growth. The number of shoots appears to correlate directly with the number and position of axillary buds (Sennerby-Forsse and Zsuffa 1995). For example, stools of *S. viminalis*, contain twice as many buds as *S. amygdaloides* Anderss. Furthermore, in *S. amygdaloides*, only the primary bud develops into a shoot. This results in the lower coppice response typical of tree willows, whereas in *S. viminalis* (a typical basket and biomass willow) the main shoot primoridia develop first, followed by two lateral primordia. The latter give rise to weaker, shorter lateral branches, which contribute to the rapid development of a new canopy but then progressively die back. As the number of buds depends on the number of remaining stem parts on the harvested stool, the initial number of shoots that sprout after harvest increases with successive rotations. A competitive hierarchy establishes during progressive cycles, with the largest stools at harvest having a larger surface area from which more shoots arise and a larger below-ground store of reserves, resulting in a higher average weight of new shoots (Verwijst 1996a). Adventitious shoot primorida can also develop without over wintering, forming sylleptic shoots along the stem during the growing season. However, these do not appear to contribute to yield in willow (Sennerby-Forsse 1995; Rönnberg-Wastljung and Gullberg 1999).

During the perennial cycle, recycling from the leaves to the stem, stool and roots is key to the provision of supplies for initial growth in the following year (see later). When the stems are harvested only the resources in the stool and root system are available and low levels of nitrogen fertilizer (approximately 60 kg/ha) are normally applied. The length of rotation and the density at which the cuttings are planted is important as both affect shoot and stool mortality rates. Planting densities of 15 000–18 000 stools per ha and a rotation cycle of 3 years are now widely adopted (Verwijst 1996a, 1996b; Kopp et al. 1997; Ledin 1998). If the harvest cycle is reduced, insufficient resources may build up limiting spring re-growth (Ceulemans et al. 1996). If densities are too high, excessive self thinning and stool mortality rates reduce yields significantly and smaller stools are out-competed by more vigorous ones (Verwijst 1996a, 1996b; Kopp et al. 1997). At optimal densities, 90% of the standing biomass in a commercial *S. viminalis* plantation is in the surviving stems and 2.3% stool mortality can be tolerated without loss in production over 30 years (Sennerby-Forsse 1995).

Studies of bud flush in *S. viminalis* have shown that an early start in spring is more important than delaying growth cessation in the autumn (Raven 1992; Rönnberg-Wastljung and Gullberg 1999). Changes in thermal time (Cannell et al. 1987) together with altering levels of cytokinins, abscisic acid (Alvim et al. 1976, 1978, 1979) and gibberellins (Junttila et al. 1988) interact in regulating dormancy, bud burst and shoot growth. The rapid mobilization of resources and simultaneous development of many shoots from each stool leads to fast build up of large leaf area, rapid growth increment and early canopy closure; an effective strategy for biomass accumulation (Sennerby-Forsse and Zsuffa 1995). Willows have indeterminate growth (Ceulemans et al. 1996) but growth cessation, as a result of changes in temperature and photoperiod, and leaf fall, are also correlated with changes in cytokinins and abscisic acid (Alvim et al. 1976, 1978). Photoperiodic ecotypes exist in *Salix* spp. in which the timing of autumn leaf abscission depends on the latitude of origin of the ecotype (Junttila 1980).

Plant canopy optimization models predict that leaf nitrogen (N) distribution in the canopy will parallel the vertical light gradient and that the ideal canopy for fast growing plants should combine high leaf area index (LAI) with a low light extinction coefficient and flat vertical leaf N gradient (Wu 1993). Based on comparisons of six varieties of willow, Weih and Rönnberg-Wastljung (2007) found that the vertical N leaf gradient showed significant negative correlation with shoot biomass production. However, canopy thickness explained only a small proportion of the variation in shoot biomass and it was suggested that changes in leaf inclination angles (with erectophile leaves in the upper canopy and more planophile leaves in the lower canopy) may also be important. Specific leaf area (SLA) also differed among genotypes but was not related with yield. Leaf traits (**Figure 1A–F**) and canopy architecture (**Figure 6**) do vary considerably among willow biomass varieties and it is possible that different strategies exist. For example, the higher yielding cultivar Tora (*S. viminalis* × *S. schwerinii*), which in the Weih and Rönnberg-Wastljung (2007) study had the weakest vertical leaf N gradient, also has a lower LAI and a more open canopy (**Figure 6**) compared with a low yielding willow reference clone (L78183) of *S. viminalis.* (Robinson et al. 2004). At least two alternative growth strategies (both associated with high yield) were identified among 32 willows by (Tharakan et al. 2005) typified by either a large number of stems (approximately 11 per stool), relatively low LAI and specific leaf area (SLA), or fewer large diameter stems (typically six per stool), high LAI and high SLA.

Mapping of QTLs in willow is slowly beginning to help unravel some of the component traits contributing to biomass yield. Not surprisingly, it is clearly a complex process but genes with major effects are being detected. Genetic variation in a number of traits, including dry matter content, bud break and growth cessation was shown to be highly additive in an 8 × 8 factorial cross of *S. viminalis* parents for all characters studied (Rönnberg-Wastljung and Gullberg 1999). Eleven QTLs affecting growth traits were identified in a Björn ((*S. viminalis* × *S. schwerinii*) × *S. viminalis*) mapping family, two of which explained 32% of the variation (Tsarouhas et al. 2002). Four QTLs affecting bud flush were also detected but these did not co-locate with any of the yield QTL (Tsarouhas et al. 2003). Yield QTL have also been mapped in K8, including one with a major effect. Co-location of stem diameter and stem height with total yield QTL was detected in addition to separate QTL for shoot number (Hanley 2003). Research is underway to resolve these QTLs more precisely and, through the poplar genome sequence, identify the possible causal genes influencing biomass yield. In addition, although studies on photosynthesis in *Salix* spp. have been limited, they have served to demonstrate that photosynthetic rates are comparable to those of herbaceous C_3 plant species (Raven 1992). More recently, components of CO_2 assimilation were investigated in a range of willow species from the NWC at Rothamsted and considerable variation identified, suggesting that scope exists for improving this trait in willow (Parry et al. 2009). These aspects, in relation to leaf and canopy traits, are being pursued further in a large collaborative program coordinated by Rothamsted (BSBEC-BioMASS: http://www.bsbec-biomass.org.uk/).

Figure 6. Canopy variation in short rotation coppice (SRC) willow; (left) var. Tora; (right) var. Ashton Stott (Stott 10).

In terms of using willow biomass for bioenergy and biofuels it is important to consider not only the total yield but also the composition of the biomass in relation to different energy conversion technologies. Compared with annual (e.g. wheat straw) and perennial grasses (e.g. Miscanthus spp.), willow biomass has generally a higher content of lignin, lower cellulose and hemicelluloses contents, a higher energy value, lower ash content and lower concentrations of problem elements such as K, Na, Cl, Si, and S (Karp and Shield 2008). These characteristics favor willow biomass for combustion, pyrolysis and gasification but suggest it is a less favorable feedstock for biological conversion to biofuels. However, substantial variation in key compositional properties relating to the quality of biomass for conversion was identified among 30 willow clones, suggesting that improvements in composition for different end-uses could be selected for (Tharakan et al. 2005). More recently, 138 genotypes of the K8 mapping population were used to examine variation in enzymatic glucose release from stem biomass and to identify QTL associated with enzymatic saccharification yield (Brereton et al. 2010). Significant variation was found in enzyme derived glucose yields and four QTLs influencing this trait were identified. These results indicate SRC willow has strong potential as a source of bioethanol and that there may be opportunities to improve the breeding programs for willows for increasing enzymatic saccharification yields and biofuel production (Brereton et al. 2010). This work is also being progressed within the BSBEC-BioMASS project referred to above.

Resource Use

Remobilization of resources during the perennial cycle is important to consider in genetic improvement because of the balance needed between maximizing harvestable yield and ensuring that sufficient reserves are in place for next years' growth (Karp and Shield 2008).

Bud burst in willow is preceded by a massive mobilization of resources. *Salix* spp. are diffuse-porous trees with heterocellular rays. Overwintered mature vessels provide the first functional vessels when growth resumes in the spring (Lawton 1976; Sennerby-Forsse 1986). The cambial activity required to produce new vessels then takes several weeks to spread slowly from the apices to the base of the stem. As the diffuse xylem vessels are relatively small, conductance of a single years' secondary xylem is not sufficient for peak transpiratory water flux and several years of secondary xylem become simultaneously functional in water movement. A flux of concentrated sugars (30 kg sucrose per m^3 in *S. fragilis*) occurs (spring sap), resulting from mobilization of carbohydrate reserves in the parenchyma cells of xylem rays in the roots (Raven 1992). Over time, as the stems grow larger and their leaves mature, they become independent of reserves from the root and eventually reserves are exported from the leaves. Willow leaves mature more slowly and show longer leaf retention compared with poplar leaves, so there is larger allocation of carbohydrates to shoot growth later in the season (Ceulemans et al. 1996). A number of genetic and genomic studies have been carried out on the timing of bud burst and growth cessation in *Populus* spp. (e.g. Bohlenius et al. 2006) including a transcriptional timetable of autumn senescence (Andersson et al. 2004). Together with the QTLs identified for these traits in willow, described above, this should help advance our understanding of the genetic control of these important developmental stages.

The biomass of willow root systems is extensive and may be equal to, or greater than, the total above-ground biomass (Isebrands et al. 1996). Despite its importance in terms of resource use, however, the root system of willow has been the subject of limited study, due to the difficulty of studying roots of mature trees in dense stands. Stott (1961) characterized the rooting pattern of *S. triandra* variety Black Maul at LARS over 6 years from initial planting. The roots systems were excavated in the first two years but from 1956 onwards a high pressure spray was used to wash the soil away – a technique requiring 1 000 gallons for the final investigation in 1960. In the first season, cuttings were characterized by numerous adventitious roots derived from root initials lying outside the phloem, and especially around nodes. Over successive years these declined in number and lateral roots at a depth of 10–20 cm dominated. Only rarely were strong vertical roots produced from the base of the cutting. Some lateral roots reached up to 3.6 m in the final year and most penetrated the root systems of other plants, indicative of potential competition.

A more recent study aimed to avoid such destructive sampling and to provide an easier means of estimating belowground biomass in willows. Cuttings of *S. viminalis* cv. Olof were maintained in pots and root development was followed using a portable capacitance meter over the course of two and a half months. Relationships between electrical capacitance (EC) and the below- and aboveground biomass were investigated. The pot findings were confirmed by excavation of the root systems of 2-year-old established trees. A strong significant linear relationship was obtained between EC and root biomass (dry weight) for the pot experiment and confirmed by results from the excavations. EC also showed good correlations with stem and leaf biomass, as well as with stem height. These results suggest that EC is a good estimator of below-ground biomass in willow and may become useful in screening genotypes for differences in root biomass traits (Pitre et al. 2010).

If SRC willow plantations are to be increasingly targeted towards "marginal" lands where nutrients and water may be limited, to reduce competition with food producing areas, stress tolerance and resource-use efficiency will be in increasingly important traits to breed for. Further research on root systems

will also contribute to this and more effort is also needed on selecting willows that can sustain yields in environments where nutrients and water may be limited.

Genetic variability has been observed among willow genotypes in nitrogen use efficiency (NUE) and water use efficiency (WUE) and both correlated with yield, although a clear effect of environmental conditions was also detected (Weih and Nordh 2002). In this same study, willow growth responses also varied, with some genotypes compensating for an initial slow start by a higher relative growth during the main growing season, whereas others grew more rapidly at first at the cost of a lower relative growth rate later on (Weih and Nordh 2002). It has also been observed that some willows allocate more to the establishment of a root system after initial planting and then grow faster after cutback (e.g. Stott 10), whilst others (e.g. Tora) allocate more to growth above ground and do not grow as fast after cut back (I.F. Shield unpubl. data, 2010). A well established root system is likely to be beneficial for resource use and these differences could be selected for.

Once the canopy is closed a significant part of the annual nutrient demands of willow are met from internal recycling, reabsoprtion from litter decomposition and atmospheric deposition. The long term nutrient requirements are restricted to the amounts lost at harvest, with between 30 and 80 kg N required, assuming a production level of 10–12 ODT/ha (Sennerby-Forsse 1995). Nitrogen response experiments in willow do not show a significant yield gain when doses exceeding this are applied (Boehmel et al. 2008). Although genetic differences in NUE could be selected for, this raises the issue as to what aspect of NUE could be targeted to achieve large gains in genetic improvement. The association mapping population in the EraNet project, mentioned earlier, has been planted on sites which are nutrient poor and may help identify genes involved in yield under limiting nutrient availability. However, further understanding of the relationships between the vertical leaf N gradient, canopy architecture (and, thus, light interception through the canopy), LAI and canopy closure (Kull et al. 1998; Kull and Kruijt 1998; Weih and Rönnberg-Wastljung 2007) will also help in the breeding of ideotypes that are more optimal in terms of N use, light interception and photosynthate assimilation and utilization.

Climate change scenarios indicate that, in addition to rising global temperatures, resulting in some areas becoming drier, higher incidences of extreme weather conditions, including spring/summer droughts, are predicted to occur. Conversely, some areas may be more prone to flooding and precipitation may increase in winter months. As willow plantations have a long lifetime of approximately 20+ years, it is important to breed varieties that can sustain yields despite fluctuations in water availability. This will also enable expansion onto a wider range of land types.

Willows are often associated with riparian habitats, leading to the common belief that they have high water use requirements and this is exploited by farmers who frequently plant willows on flood-prone land less suitable for arable crops. The rapid development of a canopy with a large LAI, while favorable for biomass accumulation, is associated with a transpiration cost, which suggests that once the canopy has closed, water may be a major limiting factor. However, willows are highly variable and species can be found that have adapted to drier conditions. These include; *S. aegyptiaca*, a species native for The Caucasus, southern Turkey, northern Iran; *S. turnorii*, *S. silicicola*, *S. relli* and *S. planifolia* which originate from the Athabasca sand dunes in northern Saskatchewan, Canada and *S. psammophila*, which originates from Mu-us, a sandy area of Inner Mongolia. These species could provide useful variation for breeding programs aimed at improving the tolerance of biomass willows to water limitation.

Previous work has shown that some genotypes can maximize WUE while ensuring stand survival even under severe drought. In a field trial of willows under two water regimes, intrinsic WUE and relative water content (RWC) varied among the clones tested and willows with higher WUE produced higher shoot biomass under conditions when water was strongly limiting (Weih and Nordh 2002; Linderson et al. 2007). In a pot experiment of four willow clones, WUE of aboveground biomass production was found to be positively correlated with the assimilation rate to stomatal conductance ratio. Increased allocation of dry matter to roots was observed in two clones and increased resistance to xylem cavitation in one of the clones, suggesting these inter-linked traits may be targeted for improvement by breeding (Wikberg and Ogren 2004; Wikbergi and Ogreni 2007). QTLs for WUE, drought-response and drought adaptation have been identified in willow (Rönnberg-Wastljung et al. 2005) but further work is required to identify the causal genes involved.

While water and nutrient limitation will become an increasing challenge for willows growing in many geographic regions in the future, willow species can also be exploited for alleviating environmental problems where nutrients and other elements are in excess (Kuzovkina and Quigley 2005). Willows have been known for some time to be effective as natural filters in reducing run-off of nutrients from agricultural land and in phytoremediation of pollutants (e.g. heavy metals and landfill leachate) (Jones et al. 2006) and effluent treatments (Aronsson and Perttu 2001). Environmental applications of willow have become increasingly important in order to respond to the need to improve organic waste handling and to meet the objectives of operational tools aimed at water protection, such as the EU water framework directive (Mirck et al. 2005). Furthermore, use of SRC in this way is more attractive to farmers as the added value that the phytoremediation confers on the energy produced has the potential to improve the economic

sustainability of the crop (Rosenqvist and Dawson 2005). There are risks associated with growing willow in high irrigation and high fertilization systems (e.g. Dimitriou and Aronsson 2004) but genetic differences in the phytoremediation potential of willows have been demonstrated (Weih and Nordh 2002; Dimitriou et al. 2006), so it should be possible to select genotypes with water and nutrient uptake physiological characteristics that are optimal for different environmental applications.

Future Perspectives

Advances in understanding the physiology and growth of willow, and in the identification of genes underlying key traits, are now at the stage where they can be used to improve the efficiency of breeding programs aimed at improving biomass willows for the bioenergy and biofuel markets. Over the past two decades important genetic resources have been established and transfer of tools and knowledge from poplar has helped considerably. Efforts so far have led to the identification of QTL for important traits for both bioenergy and biofuel end-uses. These need to be pursued to identify the underlying causal genes for use in selection programs. The location of QTL needs to be better resolved in most cases. Comparative analysis with the poplar genome sequence should provide a route for finer scale mapping and for the identification of functional candidate genes through efficient development of QTL-targeted markers. In addition, new resources such as the association mapping population now provide an alternative route to gene discovery, and comparative mapping of traits in different willow families will help identify robust QTLs. The potential usefulness of markers for carrying out trait selection in different breeding families needs to be ascertained and increased effort needs to be placed on investigating genotype × environment interactions by assessing trials on a more extensive range of sites. Further progress in willow will be accelerated as additional transcriptome, proteome and metabolome data become available and developments in sequencing technologies are fully exploited to improve our understanding of genome variation within this diverse genus.

Acknowledgements

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(Co-Editor: Martin A. J. Parry)