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Functional screening of willow alleles in Arabidopsis combined with QTL mapping in willow (Salix) identifies SxMAX4 as a coppicing response gene

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Summary
Willows (Salix spp.) are important biomass crops due to their ability to grow rapidly with low fertilizer inputs and ease of cultivation in short-rotation coppice cycles. They are relatively undomesticated and highly diverse, but functional testing to identify useful allelic variation is time-consuming in trees and transformation is not yet possible in willow. Arabidopsis is heralded as a model plant from which knowledge can be transferred to advance the improvement of less tractable species. Here, knowledge and methodologies from Arabidopsis were successfully used to identify a gene influencing stem number in coppiced willows, a complex trait of key biological and industrial relevance. The strigolactone-related More AXillary growth (MAX) genes were considered candidates due to their role in shoot branching. We previously demonstrated that willow and Arabidopsis show similar response to strigolactone and that transformation rescue of Arabidopsis max mutants with willow genes could be used to detect allelic differences. Here, this approach was used to screen 45 SxMAX1, SxMAX2, SxMAX3 and SxMAX4 alleles cloned from 15 parents of 11 mapping populations varying in shoot-branching traits. Single-nucleotide polymorphism (SNP) frequencies were locus dependent, ranging from 29.2 to 74.3 polymorphic sites per kb. SxMAX alleles were 98%–99% conserved at the amino acid level, but different protein products varying in their ability to rescue Arabidopsis max mutants were identified. One poor rescuing allele, SxMAX4D, segregated in a willow mapping population where its presence was associated with increased shoot resprouting after coppicing and colocated with a QTL for this trait.

Keywords: Arabidopsis, allelic diversity, mutant rescue, Salix, QTL, strigolactone.

Introduction
The best characterized and widely studied plant today is undoubtedly Arabidopsis thaliana, a small, short-lived, flowering annual which has been developed as a model organism for understanding the complex processes underlying plant growth and development. As research efforts intensify worldwide to meet the rising challenges of food and energy security, there is increasing interest in transferring knowledge from Arabidopsis to species of commercial relevance and particularly to those plants that are much less tractable to study. This is particularly true for many trees where studies on developmental processes can be very challenging, due to their large size, longevity and perennial growth cycle.

Willows (Salix spp.) are among the fast-growing tree species that are grown commercially as short-rotation coppice (SRC) to provide a renewable and sustainable source of biomass for bioenergy, due to their ability to produce high yields with low fertilizer inputs and their ease of propagation as vegetative cuttings (Karp and Shield, 2008; Karp et al., 2011; Volk et al., 2006). Willows were initially coppiced for basket-making, and breeding programmes aimed at genetic improvement for biomass are relatively recent. They are highly diverse, and the 350–500 species recognized (Argus, 1997) provide a rich germplasm resource for breeding. However, data on the genetic basis of many important developmental processes are limited due to the difficulty and effort required to assess phenotypic differences in the field, particularly in mature coppiced stands, and the fact that there is no robust method for transformation to validate gene function. Willows are also dioecious, highly heterozygous, and ploidy ranges from diploid to dodecaploid. A fully annotated genome sequence has not yet been published, but synteny between the willow genetic map and poplar genome has been demonstrated (Hanley and Karp, 2013; Karp et al., 2011).

Many of the target traits for improvement in willow are complex and not well understood, even in poplar, which is closely related and has been developed as a model tree (Yang et al., 2009). One such trait of fundamental importance is coppicing response, that is, the vigorous regrowth that occurs following the removal of the stems at winter harvest. Coppicing response is central to the suitability of all trees grown for biomass production in SRC cycles, as it enables sufficient biomass yield to be obtained within shorter time frames than conventional forestry (Keoleian and Volk, 2005). The number of resprouted branches also affects tree and canopy architecture and stem composition. Coppicing has also been shown to reinvigorate the plants, accelerating canopy development and shoot growth towards a theoretical maximum because of the increased number of shoots that resprout initially per coppiced stool and the changes in leaf size, specific leaf area and increased net photosynthesis associated with juvenile growth (Kauppi et al., 1988; Sennerby-Forsse, 1995; Sennerby-Forsse and Zsuffa, 1995).
Despite the key importance of coppicing response, the genetic regulation of this process with respect to the control of bud and shoot behaviour has been the subject of very limited study in trees, although genetic differences in coppicing response have been reported (Dick et al., 1998; Li et al., 2012; Sennery-Forsee and Zsuffa, 1995). Coppicing response involves the activation of pre-existing axillary buds (in the stool) that have been previously kept dormant by apical dominance, and, in contrast to trees, the mechanisms underlying these processes are very well characterised in Arabidopsis (Leyser, 2005, 2009). Of particular interest in this context are the Arabidopsis More AXillary growth (MAX) genes (MAX1-MAX4), which are known to regulate axillary bud outgrowth and branching through the production and signal transduction of strigolactones (SL) (Domagalska and Leyser, 2011).

MAX genes are conserved in both monocots and dicots. Independent identification of mutations includes max in A. thaliana, ramosus (rms) in pea (Pisum sativum), decreased apical dominance (dad) in petunia (Petunia hybrida) and dwarf (d) or high-tillering dwarf (htd) in rice (Oryza sativa) (reviewed by Domagalska and Leyser, 2011). Orthologues of MAX genes have also now been studied in kiwifruit (Actinidia chinensis) (Ledger et al., 2010) and chrysanthemum (Dendranthema grandiflorum) (Dong et al., 2013; Liang et al., 2010).

Currently, six members of the SL pathway have been identified. MAX3/RMS5/HTD1/D17/SICCD7 encodes CAROTENOID CLEAVAGE DIOXYGENASE 7 (CCD7) (Booker et al., 2005; Johnson et al., 2006; Vogel et al., 2010; Zou et al., 2006) and MAX4/RMS1/DA1/D10 encodes CCD8 (Booker et al., 2007; Snowden et al., 2005; Sorefan et al., 2003). Both are involved in the production of SLs (Arite et al., 2008; Auldridge et al., 2006; Booker et al., 2004; Schwartz et al., 2004), and in Arabidopsis function up stream of MAX1, a cytochrome P450, CYP711A1 (Booker et al., 2005; Scaffidi et al., 2013). D27 (Lin et al., 2009) and D14/DBB/HTD2 (Arite et al., 2009; Gao et al., 2009; Liu et al., 2009) have been identified in rice and recently analysed in Arabidopsis (Waters et al., 2012a, b). While the iron-containing protein D27 is involved in SL biosynthesis (Aldre et al., 2008), the α/β-fold hydroxylase D14/DBB/HTD2 binds and cleaves strigolactone and may be involved in its perception and/or processing (Hamiaux et al., 2012; Kagiyama et al., 2013). MAX2/RMS4/D3 is an F-box protein involved in SL signal transduction (reviewed by Domagalska and Leyser, 2011).

We previously demonstrated that strigolactone affects willow bud activity in a very similar manner to Arabidopsis (Ward et al., 2013). Furthermore, transformation rescue of Arabidopsis max mutants can be used as a platform to identify functional variation between willow MAX alleles (Ward et al., 2013). In this present study, this approach was exploited to identify allelic variation underlying coppicing response. A large Arabidopsis mutant rescue screen was performed to test a series of 45 Salix MAX orthologous (SxMAX1-SxMAX4) alleles for putative functional variation. With a view to enabling downstream genetic analysis if required, the alleles included were cloned from parents of large willow mapping populations generated from crosses made based on phenotypic differences in shoot number and branching architecture. Here, we report the results of the mutant rescue screen, and the downstream analysis of alleles highlighted as potentially interesting. Segregation of one such allele, SxMAX4D, was subsequently found to be associated with the number of shoots that resprouted after coppicing in willow. These results demonstrate the power of using Arabidopsis to test large numbers of allelic variants for the identification of useful variation for further study in more targeted resource-intensive, field-based studies in trees.

Results

Isolation of willow SxMAX alleles

In total, 47 SxMAX alleles encoding unique protein products were cloned from the 15 parents of 11 willow mapping populations (Table 1). For all four SxMAX loci, the majority of genotypes screened yielded two DNA sequences per diploid genome, with the exception of SxMAX1 in parental lines S3, RES0453, RES0506 and RES0628; SxMAX2 in S3, RES0453 and RES0506; SxMAX3 in RES0655; and SxMAX4 in S3, RES0655 and RES0663, for all of which only one allele was amplified. From partial sequencing data, SNPs were discovered in SxMAX3 from RES0615, RES0506 and RES0501, but only one of the two alleles was successfully cloned and analysed in this study. No SxMAX3 alleles from RES0099, RES0432, RES0453 RES1059, or SxMAX4 alleles from RES0432 were cloned here due to project time constraints. Where only one allele was identified, it is likely that the Salix clone from which the genomic DNA was extracted is homozygous at that locus. This is particularly likely for S3, for which no other heterozygous markers have been identified in this genome region in previous studies in the K8 mapping population. In other cases, for example, RES0506 where only one allele was obtained for SxMAX1 and SxMAX2, multiple amplification attempts, and the use of Salix primers designed to regions conserved among all other amplified Salix species, did not result in the discovery of a second allele. However, due to the potential divergence between the allele sequences within RES0506 (considered to be S. caprea L. x S. cinerea L. x S. viminalis L), the possibility that a second allele failed to amplify under the range of conditions used cannot be completely ruled out.

Sequence diversity within willow MAX genes

Analysis of the predicted coding sequences (CDS) for each locus revealed SNP frequencies of 33.3, 74.3, 29.2 and 53.8% SNPs per kb for SxMAX1, SxMAX2, SxMAX3 and SxMAX4, respectively, and indicated that, on average, approximately one-third of the SNPs detected would result in nonsynonymous amino acid substitutions (Table 2). SxMAX2 alleles contained proportionately 30% or more SNPs than the other three loci (Table 2). Analysis of CDS alignments (data not shown) and phylogenetic trees (Figure 1) revealed that polymorphisms within all four loci were not evenly distributed between species but were concentrated within particular alleles. For example, alleles from S. triandra contributed a greater proportion of allelic difference to the loci as a whole, as revealed by their distance relative to other alleles in SxMAX1, SxMAX2 and SxMAX4 CDS trees (Figure 1a, b and d). The separation of this species from the others indicated by these results is in agreement with previous Salix molecular diversity data reported by Trybush et al. (2008) as was the consistency of the allele clustering in accordance with current Salix taxonomic thinking. The latter was also true for alleles of hybrid genotypes, which were assigned to separate clusters based on their parentage. For example, alleles from RES0789 (a S. viminalis x S. purpurea hybrid) were consistently assigned to different groups as expected (Figure 1a, b and d).

The inferred protein sequences of the Salix alleles aligned with the orthologous Populus trichocarpa sequences for SxMAX4 are
shown in Figure 2, while sequences for the SxMAX1-3 loci are provided in Figure S1. The willow MAX alleles were 98%–99% identical, but a number of amino acid changes were identified (Figure 2 Table 2, Figure S1). Conserved positions in synapomorphic regions found in MAX2, MAX3 and MAX4 by Challis et al. (2013) were also conserved in all the Salix alleles. There were also no differences within the Salix alleles in any residues that correlated with previously published mutations, nor the proposed substrate specificity residues of MAX3 and MAX4 (Delaux et al., 2012). Nonconservative changes were identified at three amino acid sites (Figure 2) of which two were unique and therefore of potential interest. SxMAX4D (S. viminalis) has an alanine (neutral–nonpolar) to aspartic acid (acidic) substitution at position two. This alanine is highly conserved in other genera for which MAX4 protein sequence is available (Challis et al., 2013). SxMAX4E (S. aurita) has an arginine (basic) to serine (neutral) substitution at position two of SxMAX4G (an allele able to rescue fully max4-1) has an arginine (basic) to serine (neutral) substitution at position two of SxMAX4G (an allele able to rescue fully max4-1). (Figure 3). Multiple independent transgenic lines were assayed for each construct to account for the fact that different transgenic lines carrying the same construct can show quantitative differences, due to transcriptional or post-transcriptional effects (Schubert et al., 2004). Branching data from lines containing MAX4B, E and G have previously been published in Ward et al. (2013) but are included here again for comparison.

All of the SxMAX1 and SxMAX3 constructs were able fully to rescue max1 and max3 shoot branching to a wild-type level, and little variation was observed between the independent transgenic lines (Figure 3). In contrast, a great deal of variability in rescue ability was observed between independent transgenic lines transformed with SxMAX2 constructs (Figure 3). For each construct, however, at least one line was able to restore branching of max2-1 to wild-type levels (significance tested by Tukey test), demonstrating that they all had the ability to complement the max2 phenotype. Of the seventeen SxMAX4 constructs transformed into max4-1, fifteen showed wild-type branching among the majority of the independent transgenic lines (Figure 3). All five of the SxMAX4E lines and four of the five SxMAX4D lines presented in Figure 3 (which had unique amino acid differences at positions 204 and 2, respectively) only partially reduced max4-1 branching.

To test whether the substitution of the conserved alanine at position two to aspartic acid in SxMAX4D was responsible for the partial rescue of max4-1 branching by the SxMAX4D allele, a number of constructs were made in which: (i) the alanine at position two of SxMAX4G (an allele able to rescue fully max4-1) were taken to homozygosity and assayed. The data from five lines are shown, representing the full range of phenotypes observed (Figure 3). Multiple independent transgenic lines were assayed for each construct to account for the fact that different transgenic lines carrying the same construct can show quantitative differences, due to transcriptional or post-transcriptional effects (Schubert et al., 2004). Branching data from lines containing MAX4B, E and G have previously been published in Ward et al. (2013) but are included here again for comparison.

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| DNA source | Parent | Clone name | MAX1 | MAX2 | MAX3 | MAX4 |
|------------|--------|------------|------|------|------|------|
| S3         | S. viminalis L. x (S. viminalis L. x S. schwerinii) | S3 | B, B | A, A | A, A | B, B |
| R13        | S. viminalis L. x (S. viminalis L. x S. schwerinii) | R13 | B, C | A, B | A, A | B, B |
| RES0099    | S. triandra L. | ‘Semperflorens’ | G, F | O, P | * | O, Q |
| RES0432    | S. daphnoides Vill. | Fastigate | B, B | G, H | * | * |
| RES0453    | S. aurita L. | – | D, D | I, I | * | E, F |
| RES0506    | S. caprea L. x S. cinerea L. x S. viminalis L. | – | B, B | A, A | J,* | M, N |
| RES0615    | S. schwerinii Wolf | K3 Hilliers (WB 50 0 354) | B, C | J, K | I,* | R, S |
| RES0627    | S. viminalis L. x S. schwerinii Wolf | 910006 ‘Bjorn’ | B, C | A, M | A, H | B, U |
| RES0628    | S. viminalis L. x S. schwerinii Wolf | 910007 ‘Tora’ | C, C | A, N | A, F | B, T |
| RES0655    | S. viminalis L. | Stone Oser | B, B | A, D | D, D | B, B |
| RES0663    | S. viminalis L. | Pulchra Ruberrima | B, B | A, A | A, A | B, B |
| RES0674    | S. viminalis L. | English Rod | B, B | A, D | A, A | B, D |
| RES0789    | S. purpurea L. x Ulbrichtweide | – | B, E | A, E | E, G | G, H |
| RES0901    | S. x albertii (S. integra x S. suchowensis) | 42/17 | H, H | E, E | K,* | J, K |
| RES1059    | S. x fresiana (S. viminalis x S. repens) | – | B, B | B, L | * | B, L |

*Indicates not known.
was mutated to an aspartic acid (SxMAX4G+); (ii) the aspartic acid at position two in SxMAX4D (an allele unable to rescue fully max4-1) was mutated to alanine (SxMAX4D-); and (iii) the alanine at position two in Arabidopsis MAX4 (which can fully rescue Arabidopsis mutant max4-1 (Sorefan et al., 2003) was mutated to aspartic acid (AtMAX4D). All three constructs were transformed into max4-1, and ten homozygous independent lines tested for their ability to rescue the highly branched max4-1 phenotype. Lines expressing SxMAX4D- (aspartic acid changed to alanine) were able fully to rescue max4-1 branching in an increased number of lines (compare Figure 3 with Figure 4c). In the case of AtMAX4D (alanine changed to aspartic acid), none of the ten independent transgenic lines studied were able fully to rescue max4-1 (Figure 4c). These data suggest that the alanine at position 2 is important for MAX4 function. However, SxMAX4G+ (alanine changed to aspartic acid) was unchanged in its ability to restore wild-type branching as compared to SxMAX4G (compare Figure 3 with Figure 4a), indicating that while important, the alanine is not essential in some allelic contexts.

**Functional relevance in willow**

One of the available willow mapping populations, mpA, has a suitable genotypic constitution (expected 1:1 pseudo testcross segregation) to test whether any copiquing phenotypes corresponded with SxMAX4D segregation. From this population, 100 genotypes were randomly selected and assessed for resprouting from the coppiced stool. After excluding the possibility of spatial trends in the field data, a significant difference in the number of resprouted stems was detected in this population according to the presence/absence of the SxMAX4D allele (P < 0.01; Student’s t-test) (Figure 5). Subsequent QTL analysis by interval mapping detected a significant QTL on paternal (RES0674) linkage group VI that colocated with segregating alleles SxMAX4B and SxMAX4D (Figure 6). The QTL explained 8.2% of the phenotypic variance. At the population level, plants heterozygous for SxMAX4B and SxMAX4D alleles produced a greater number of stems than those homozygous for SxMAX4B.

**Discussion**

**Allelic diversity in the mapping populations**

Our data provide a detailed survey of allelic diversity at the SxMAX1, SxMAX2, SxMAX3 and SxMAX4 loci in 15 parents of Salix mapping populations used in the willow breeding programme at Rothamsted Research, UK. Currently, breeders of biomass willow in Europe tend to select lines containing S. viminalis, and the Rothamsted populations reflect this bias (Table 1). There are few data available on allelic diversity in willow breeding stocks, but, within the set used here, our data indicate that SNPs were present at a frequency of between 29 and 74 per kb in the exons of the MAX genes. Although several species other than S. viminalis are present in this set, either as pure species or in the pedigrees of the bred lines, the majority of the diversity found arose from the divergence of S. triandra RES0099 from the other species (as highlighted by a distinct node in the CDS phylogenetic trees in Figure 1). This is consistent with the findings of Trybush et al. (2008), who first noted the separation of S. triandra from a wide range of other Salix species. Our findings are within a range reported for Salix exons in a recent study in which between 8 and 96 SNPs/kb were detected among 12 genes involved in wood formation for a diverse range of Salix taxa (Perdereau et al., 2013). In poplar, from 8 to 105 SNPs were detected per kb, depending on the species and genes investigated (see also Perdereau et al., 2013). These data suggest that SNP frequencies are generally high for the Salicaceae, in comparison with those for ESTs of wheat (16.5) and maize (8.9), for example (Barker and Edwards, 2009). The genus Salix comprises a large range of diverse species, over 10-fold more than Populus. MAX genes are conserved among genera, but our findings suggest there is a high probability of uncovering further novel allelic diversity of use to breeders if a wider number of species is examined.

We found considerable variation between the genes examined in the extent of allelic diversity. The short branch lengths of the MAX1 phylogenetic tree indicate that the SxMAX1 alleles contain between them fewer base substitutions per site relative to the other three loci in these willow species. In contrast, even taking into consideration the longer CDS for SxMAX2, alleles at this locus contained between them at least 30% or more SNPs than the other three loci. However, in the Salix individuals studied here, the diversity occurred within relatively few alleles compared with the other loci, such that total numbers of alleles were not higher for SxMAX2 than SxMAX4, for example, where polymorphisms were more evenly spread.

**Functional significance of allelic variation**

In a proof-of-principle study, we have previously shown that it is possible to detect functional allelic variation in the coding sequence of willow MAX genes using transformation rescue of the cognate Arabidopsis mutant. The data presented in this study provide a much more extensive survey of the functional allelic variation.

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**Table 2** Inferred CDS and protein information

| Gene       | MAX1 | MAX2 | MAX3 | MAX4 |
|------------|------|------|------|------|
| Number of DNA sources | 15   | 15   | 11   | 14   |
| CDS length (bp) | 1593 | 2085 | 1842 | 1845 | 1674 |
| Total number of SNPs per alignment | 53   | 155  | 54   | 90   |
| SNP frequency (SNPs per kb) | 33.3 | 74.3 | 29.2 | 53.8 |
| Number of 3 bp INDELs | 0   | 0   | 3   | 0   |
| Alleles at inferred CDS level | 16   | 22   | 11   | 21   |
| Number of inferred protein sequences | 7   | 14   | 9   | 17   |
| Protein length (amino acids) | 530 | 694 | 613 | 614 | 615 | 557 |
| Predicted% Identical Sites | 97.0 | 92.5 | 96.3 | 94.4 |
| Predicted Protein% | 99.5 | 98.6 | 99.1 | 98.9 |

*Including RES0675, RES0506, RES0501 from which only one of two alleles are known and excluding RES0099, RES0452, RES0453 and RES1059.

†Excluding INDELS.

‡1846.5 bp average length was used to calculate SNP frequency for MAX3.

§Excluding 3 bp in Del.
variation in the MAX gene coding sequences of the Rothamsted mapping population parents. Of the 45 alleles tested, only two showed evidence of consistently poor rescue, implying that most of the allelic diversity in the alleles does not affect gene function to the degree detectable in this assay. The sensitivity of the assay is difficult to judge, but it is important to note that the degree of variability between the independent transgenic lines was highly dependent on the transgene, which will affect the ability to detect functional allelic diversity above the noise of variability in transgene activity. It is therefore possible that some functional diversity, for example among the MAX2 alleles, was missed. At the opposite end of the spectrum, it is possible that very low levels of activity from, for example MAX3, are sufficient for full rescue, which might also reduce the sensitivity of our assay. These factors make it likely that not all the functionally important variation in the alleles was detected in this study, and certainly, transforming with coding DNA from start to stop codons only, as carried out here, would be unlikely to identify polymorphisms affecting expression level. Nonetheless, it is interesting that the alleles showing evidence of reduced function were those with nonconservative substitutions in conserved amino acids, suggesting that this could be used to prioritise allele testing with relatively low risk of missing important variation.

MAX4D
The results obtained here suggest that the presence of an aspartic acid residue at position two, or the absence of an alanine, can reduce the ability of MAX4 to function. The consensus residue at position two of the Populus trichocarpa orthologues is an aspartic acid, which is conserved across the Salix MAX gene family. The presence of an alanine at this position is rare, and may explain the reduced function of MAX4D in our assay.

Figure 1 Consensus Trees of Salix MAX genes CDS with respective Populus trichocarpa orthologues. Rooted Bootstrap Consensus Trees (50% majority rule consensus) of Salix MAX genes CDS with P. trichocarpa orthologues used as the out-groups and bootstrap support node labels, generated from MUSCLE alignments (constructed using MUSCLE software available in Geneious Pro 5.5.6 (Drummond et al., 2011) set to default settings, only SxMAX3 needed minor manual adjustment so that clearly misplaced positions were more appropriately aligned) using Geneious Tree Builder software set to Jukes-Cantor; Neighbour-Joining; Bootstrap Resampling with 100 replicates, also available in Geneious Pro 5.5.6 (Drummond et al., 2011). (a) MAX1, (b) MAX2, (c) MAX3, (d) MAX4.
Figure 2  Salix and Populus trichocarpa orthologous protein alignments for MAX4. Inferred CDS were translated using Geneious Pro 5.5.6. (Drummond et al., 2011). The inferred protein sequences of the SxMAX alleles were then aligned with the orthologous P. trichocarpa protein sequence using the MUSCLE algorithm within Geneious Pro 5.5.6 with default settings. Nonconsensus residues were highlighted according to Geneious Pro 5.5.6 default settings.
this position is alanine, and when it is replaced with aspartic acid, as in SxMAX4D, ability to rescue the Arabidopsis max4-1 is reduced, while substituting the aspartic acid for an alanine, thereby restoring the consensus, improves the ability of the allele to rescue. Furthermore, a mutated Arabidopsis MAX4 protein in which the alanine to aspartic acid change was replicated could no longer fully rescue the Arabidopsis max4-1 branching phenotype. Taken together, these data suggest that the alanine at position 2 is worthy of further study with respect to MAX4 function. However, changing the same alanine in SxMAX4G to aspartic acid had no effect on the ability of the resulting protein to rescue the max4-1 branching phenotype. Examination of the protein sequences (Figure 2) shows additional changes are present in SxMAX4G compared with SxMAX4D, suggesting that the alanine is not required and/or the aspartic acid is not detrimental in all allelic contexts.

**MAX4D and the willow coppice response**

Previous studies have shown that mutant lines of Arabidopsis can be used to test functional differences between orthologous genes from commercially important plants based on their ability to rescue cognate mutant lines (Ward et al., 2013). This study progresses this a further step and associates the allelic differences with regulation of a complex trait. The MAX4D allele was found to be associated with increased resprouting after coppicing, and the MAX4 locus was found to map within a QTL for this trait. These data suggest that a component of variation for a highly complex trait has been resolved to the level of single candidate quantitative trait nucleotide (QTN) – a step rarely achieved in quantitative genetic studies in crop plants.

Prior to this study, a genetic basis to coppice response had not been identified. The finding that the MAX4 locus plays a role now enables manipulation of this trait, which has diverse relevance in terms of applications. Coppicing reinvigorates growth and an improved coppicing response would be beneficial for improving productivity of SRC systems (Kauppi et al., 1988). Stems in coppiced stands also have higher bark proportions compared with single stem systems as the bark is roughly proportional to the area: volume ratio of the stem (Adler et al., 2008). This has relevance for the use of wood chip in thermal conversion technologies where

![Figure 3](image-url)
alkali metals (e.g. K and Na) in the bark can cause problems and where the nitrogen or sulphur containing compounds form NOx or SOx in the exhaust gases. Conversely, bark is a source of extractives comprising many diverse compounds (Kammerer et al., 2005) and may come to have value in biorefining as source of valuable bioproducts and/or industrial chemicals. Manipulation of bark proportions by managing the numbers of shoots that resprout after coppicing provides a novel route to optimizing SRC feedstock for different industrial uses. Selections based on allelic diversity at MAX4 will now be incorporated into the breeding programme at Rothamsted, UK.

Previous research on coppicing in willow has investigated the histology of bud activation (Sennerby-Forsse and Zsuffa, 1992, 1995) and focussed on applied aspects, mostly on finding the optimal balance between the positive effects of coppicing and the negative effect of leaving insufficient time between harvests for the build-up of remobilised reserves in the stool and roots (Verwijst, 1996a,b). It is well known that shrubby willows (subgenus Vetrix) respond more vigorously to coppicing than tree willows (subgenus Salix), suggesting a genetic basis to this trait. However, now that the importance of MAX4 has been identified, future studies can focus on understanding the role of strigolactones in coppicing response and on determining the mechanisms regulating final shoot numbers in coppiced stools.

Success in mapping a QTL for coppicing response in this study was dependent on the method of scoring the phenotype with respect to number of shoots. Previous studies have reported QTL for stem traits (Hanley and Karp, 2013; Tsarouhas et al., 2002), but shoot number in willow can be difficult to resolve by QTL mapping because it is normally scored at the end of each year and/or at harvest (i.e. at the end of the 3-year coppice cycle). However, the shoot number of mature willow plants is not simply the product of the number of shoots that initially sprouted from the stool. Self-thinning of the sprouted shoots also occurs in which many buds sprout simultaneously, and the resultant stems are then progressively thinned to a lower number. This self-thinning process is thought to involve differential growth rates, dominance and suppression of stems and re-allocation of resources (Sennerby-Forsse et al., 1984). The proportion of buds that sprout and the degree of self-thinning both vary among genotypes, but the nature of the relationship between them has yet to be resolved. In recognition of the complexity of this trait, the approach used here was to count the number of shoots that sprouted from the cut-stool as an indicator of coppicing response instead of shoot number at harvest. Studies aimed at understanding the relationship between self-thinning and the proportion of activated and sprouted shoots are now underway.

Willow has been the focus of the current study, but the results on coppicing response have relevance to other tree species,
Figure 6  A significant QTL for shoot number production after coppicing colocalizes with segregation of the SxMAX4D allele. The parental linkage map spanning chromosome VI for S. viminalis genotype RE50674 is shown. Markers are SNPs or microsatellites (prefixed SB- or SV-). Markers names beginning with 6g represent orthologous genes in the Populus genome sequence (www.phytozome.net). The QTL locates with segregating SxMAX4 and SxMAXD alleles, which also showed differential mutant rescue capability in Arabidopsis maxd mutant lines. The QTL length reflects a 1-LOD decrease from the peak position and excludes SxMAX1. The chromosomal significance threshold is indicated by the dashed line.

including poplar, and the approach adopted could be applied to investigate other candidate regulators of agronomically important complex traits. Genetic crosses are resource intensive to pheno-

Figure 5  Testing functional relevance in a willow mapping population. (a) Boxplot showing significant difference (t = –2.76, P = 0.007; Student’s t-test) between SxMAX4 BB and BD classes for the number of resprouting shoots postcoppice (mean BB 59.10, mean BD 71.31; SED = 4.419; d.f. = 98) for 100 randomly selected individuals of the mpA willow mapping population. (b) Frequency distributions for shoot number postcoppice for BB and BD classes.

Materials and methods
Willow material
The Salix plants comprised a set of confirmed diploid genotypes from the NWC that were chosen on the basis of phenotypic differences in branch number and architecture as parents of mapping populations for a wide diversity of trait studies. The populations were planted between 2008 and 2009 and are maintained at Rothamsted Research (RRs). Details on the genetic background of these genotypes are provided in Table 1 and in Hanley and Karp (2013). Genomic DNA was extracted using DNeasy Plant extraction kit (Qiagen, Crawley, UK).

Isolation of the Salix MAX genes
Putative orthologues of the Arabidopsis MAX1 (At2 g26170), MAX2 (At2 g42620), MAX3 (At2 g44990) and MAX4 (At4 g32810) genes were identified in the Populus trichocarpa v2.2 genome sequence (http://www.phytozome.net) as described in Ward et al. (2013). Salix orthologues of the P. trichocarpa MAX1 (POPTR_0006s24320), MAX2 (POPTR_0014s13910), MAX3 (POPTR_0014s05590) and MAX4 (POPTR_0006s25490) genes were then amplified, initially from the diploid genotype R13 [S. viminalis x (S. viminalis x S. schwerinii); (Hanley et al., 2006)], using primers designed to predicted P. trichocarpa coding sequence (CDS). Resulting PCR products were gel-purified (QIAquick Gel Extraction Kit, Qiagen), sequenced and mapped on a P. trichocarpa-anchored Salix map (KB; Hanley et al., 2006) to confirm orthology (Ward et al., 2013). Salix sequences were assembled using ContigExpress in Vector NTI 10.1.1 (Life Technologies Ltd, Paisley, UK).

SxMAX alleles were cloned from the set of diploid Salix genotypes that are parents of mapping populations maintained at RRs (Table 1). Amplifications used genomic DNA as a template and Salix-derived primers (Table S1). For S3 and R13 (parents of the KB mapping population), primers designed to the 5′ and 3′ CDS ends of each gene were used. To account for known polymorphisms at equivalent CDS priming sites in the other genotypes studied, primers were designed outside the CDS for SxMAX1, SxMAX2 and SxMAX4 (Table S1). Increased diversity in the SxMAX3 upstream sequence prevented use of a common primer in this region. Instead, several genotype-specific 5′ CDS
primers were used in conjunction with a common 3' CDS primer (Table S1). AccuPrime Pfx SuperMix (Invitrogen) was used to generate PCR products that were gel-purified, cloned into the pENTR/D-TOPO vector (Invitrogen) and sequenced to obtain coverage of all exons. To ensure sequences analysed were true to the genomic DNA, either multiple individual clones were sequenced, or clones were compared with corresponding genomic sequence generated by direct sequencing of PCR products or both. BLASTN searches against the P. trichocarpa v2.2 genome sequence were used to ensure that all cloned sequences showed a best hit to the original P. trichocarpa orthologous gene target and not a paralogous sequence.

Sequence analysis

Populus trichocarpa v2.2 gene models (www.phytozome.net) were used to infer SxMAX CDS sequences. MAX2 has no introns in any species so far examined. Examination of the predicted splice sites for MAX1, MAX3 and MAX4 P. trichocarpa indicated that, with the exception of one intron within MAX3, the terminal GT and AG residues of all predicted introns were fully conserved between Salix and P. trichocarpa. The discrepancy in MAX2 (in which the A of the predicted P. trichocarpa 3' AG splice site of intron six is consistently a G in Salix) suggested that the predicted P. trichocarpa gene model may be incorrect, with an extra intron predicted. Although a paucity of public Salix or Populus mRNA/EST sequences for this gene prevented a more definitive analysis, comparison with MAX3 orthologous proteins from several other plant genera also suggested the extra P. trichocarpa intron is erroneous. Therefore, cDNA of this region of P. trichocarpa was sequenced and did not contain the sixth intron, confirming the P. trichocarpa v2.2 prediction was erroneous. The CDS sequence based on a P. trichocarpa MAX3 gene model, but omitting the predicted sixth intron annotation, was used throughout this study.

Nucleotide sequences were aligned using the MUSCLE algorithm within Pro 5.5.6 software (Drummond et al., 2011; Kearse et al., 2012) using default settings. Only MAX3 required some minor manual adjustment. Alignments were then scored for synonymous and nonsynonymous SNPs. The MUSCLE alignment for each MAX locus was used to construct a rooted phylogenetic tree with the corresponding P. trichocarpa CDS as an out-group (Geneious Tree Builder default settings; cost matrix: 65% similarity (5.0/-4.0), Genetic Distance Model: Jukes-Cantor, Tree build Method: Neighbour-Joining; Bootstrap Resampling 100 replicates).

Inferred CDSs were translated using Geneious Pro 5.5.6. (Drummond et al., 2011). The inferred protein sequences of the SxMAX alleles were then aligned with the orthologous P. trichocarpa protein sequence, again using the MUSCLE algorithm within Geneious Pro 5.5.6 with default settings. Only SxMAX3 needed minor manual adjustment.

Generation of transgenic Arabidopsis

The SxMAX alleles were transferred from pENTR/D-TOPO to the GATEWAY compatible binary destination vector pK7WG2 (Karimi et al., 2002) using LR Clonase II Enzyme Mix (Invitrogen) and transformed into the corresponding Arabidopsis max mutant background via Agrobacterium tumefaciens strain GV3101 using the floral dip method (Clough and Bent, 1998).

All Arabidopsis max lines are in the Columbia-0 background. For transformation, max1-1, max2-1, max3-9 and max4-1 plants were grown in 8-cm square pots containing F2 compost treated with Intercept 70WG (both from Levington Horticulture, Ipswich, UK). The plants were grown in a growth room at 21 °C with 16 h light, 8 h dark and a light intensity of 100–120 μmol/m²s².

Transformants were selected on agar solidified ATS medium containing 50 μg/mL kanamycin (Sigma, Poole, UK). For each construct, at least 10 independent single insertion lines were taken to homozygosity.

Shoot branching assay

A decapitation assay was used to quantify branching (Greb et al., 2003; Ward et al., 2013) in which the total number of rosette branches produced following decapitation were scored. Plants grown in P40 multitrays (Desch Plantpak Ltd, Maldon, Essex, UK) in F2 compost as above. Five of the ten independent single insertion lines studied have been chosen to reflect results for each allele and are presented graphically.

Mutagenesis

To produce the SxMAX4D- construct, the aspartic acid at position two in SxMAX4D was mutated to alanine by amplification from the pENTR/D-TOPO vector containing SxMAX4D using the forward primer CACCATGGCTTCCTTGCCATTTC, which replaced the A at CDS base five with a C, in conjunction with the common reverse primer located outside of the Stop codon GATAGCTAATACACAAACCCC. The SxMAX4D+ construct was generated using the same approach, but by amplification from the SxMAX4G construct using the forward primer CACCATGAGTCCTTGGCCATTTTCC which replaced the C at CDS base five with an A and therefore an alanine at position two with an aspartic acid. The common reverse primer GATAGCTAATACACAAACCCC was used. The mutated alleles were fully sequenced for verification as described in ‘Isolation of the Salix MAX genes.’

To change the alanine at position two to aspartic acid for the generation of an Arabidopsis based version of SxMAX4D (AtMAX4D), the CDS of MAX4 was amplified from Columbia cDNA using the forward primer ATCCCAGGATGGATTCTTTGA TCACACAC which replaced the fifth base of the CDS with an A instead of a C, and reverse primer GATCTAGATTAATCTTTGGGA TCCAGCA. Restriction sites are present in the primers to clone the AtMAX4D amplicon behind the 35S promoter in the pART7/pART27 vectors (Gleave, 1992).

QTL analysis

Segregation of SxMAX4D was tested for phenotypic association with coppicing response in willow mapping population A (mpA), which was planted in a field experiment at Rothamsted Research, Harpenden, Hertfordshire, UK, in March 2008. The male parent of the mpA population (S. viminalis RES0674) is heterozygous for SxMAX4D and SxMAX4B alleles, while the female parent (KB-411) is homozygous SxMAX4B. Following establishment growth after planting in Spring 2008, plants were first coppiced in February 2009 and then again in February 2011 after 2 years’ uninterrupted growth. In Spring 2011, the total number of stems resprouting from stools was recorded for a random set of 100 mapping population individuals and 11 clonal replicate controls (genotype KB-411) regularly spaced throughout measured area. Mixed modelling was used to test for any spatial trends across the field site but none were found. Trait data were tested for association with SxMAX4 genotype using a Student’s t-test. QTL analysis was then used to determine the chromosomal position of the underlying variation. A genetic map of chromosome VI was
generated by screening a set of previously developed microsatellite and SNP markers against 384 mpA progeny, using genotyping methods previously described by Hanley et al. (2006). Interval mapping within MapQTL 4.0 software (Kyeza@, Plant Research International, Wageningen, the Netherlands) was used for initial QTL analysis. To maximize resolution once a rough QTL position was identified, additional SNP markers in the vicinity of SxMAX4 were developed and mapped as before, and the QTL analysis repeated. A chromosomal QTL significance threshold was determined by permutation test (1000 permutations).

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**Supporting information**

Additional Supporting information may be found in the online version of this article:

**Figure S1** Salix and Populus trichocarpa orthologous MAX protein alignments for MAX1-3.

**Table S1** Salix MAX cloning information.