Aspects of Applied Biology 90, 2008
Biomass and Energy Crops III

Overcoming barriers to crossing in willow (*Salix* spp.) breeding

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Summary

A *Salix* breeding programme, BEGIN, has been running since 2003 at Rothamsted Research. Over 300 crosses have been made during this period. The breeding programme is focused on producing high yielding, pest and disease resistant elite genotypes that are optimal for UK environmental conditions.

Crossing success rate has risen from a low of 37.5% to a high of 56.4% in 2008. Using flow cytometry to estimate ploidy levels has increased the efficiency of crossing by 28%. Ploidy estimates of key germplasm, cultivars, breeding progeny and key species are presented for those of greatest interest to *Salix* breeders.

An embryo culture protocol for *Salix* is presented which successfully rescued embryos as young as 5 days post pollination and proved robust at 8 days post pollination. Other techniques to overcome crossing barriers are also discussed.

Key words: *Salix*, crossing barriers, embryo culture, flow cytometry

Introduction

Interest in dedicated perennial energy crops (short rotation coppice willow and miscanthus) has grown enormously in the past 20 years. Most recently there has been a realisation that they offer greater greenhouse gas and energy efficiencies and flexibility to be planted on a wider range of land types than annual arable crops for energy.

Rothamsted Research runs the Biomass for Energy Genetic Improvement Network (BEGIN), the UK's only willow breeding programme. In the five crossing seasons between 2004 and 2008 313 crosses have been attempted. Breeding efforts are underpinned by one of the largest and most comprehensive willow germplasm collections in the world, the UK National Willow Collection (NWC). The NWC, which was initiated in the 1920s at Long Ashton Research Station, moved to Rothamsted Research in 2002, it contains in excess of 1300 accessions and comprises of over 100 different *Salix* species (Trybush *et al*., 2008). BEGIN started in 2003 and furthered the work of the European Willow Breeding Programme (EWBP) detailed in Lindegaard & Barker (1997). BEGIN has continued performing crosses to broaden the species range of commercial varieties, which had been dominated by *Salix viminalis* and *S. viminalis* × *S. schwerinii* hybrids.

Worldwide, other willow biomass breeding programmes include significant efforts in Sweden (Ahman & Larsson, 1994; Larsson, 1998) ongoing since 1987 and North Eastern USA (Kopp *et al*., 2001) ongoing since the late 1990s. Despite the attention that the genus has received,

it has been in domestication for biomass purposes for a small amount of time compared with conventional agricultural crops and there is still potential to improve biomass yields substantially through plant breeding.

Early work on *Salix* concluded that there was widespread hybridisation between species (Heribert-Nilsson, 1918). This combined with their dioecious nature and their ability to reach sexual maturity within two years has led to the belief that willows are very amenable to breeding. This is only partly true, however, as crossing barriers have been encountered. Later authors unanimously concluded that interspecific hybridisation in nature had been hugely overestimated (Argus, 1974; Dorn, 1976; Skvortsov, 1999). Crossing barriers that prevent interspecific hybridisation in nature include non-synchrony of flowering and ecological separation.

Flow cytometry

Detecting sterile clones using flow cytometric techniques to estimate the ploidy of parental material offers the chance to improve crossing success and efficiency. *Salix* has a basic chromosome number of 19 (Karp & Shield, 2008). *Circa* 40% of the species in the *Salix* are polyploid (Suda & Argus, 1968). Polyploids range from triploids to the atypical dodecaploid *S. maxxaliana* with 2n=190 (Zsuffa *et al*., 1984). Identifying triploids (3n) and pentaploids (5n), which are sterile or have severely reduced fertility is essential when selecting parental material for breeding. Screening is necessary as different ploidy levels have arisen within species and often show no taxonomic differences (Zsuffa *et al*., 1984).

Embryo culture techniques

Premature abscission of pistillate catkins, early dehiscence of capsules or poor germination caused by a large amount of immature seeds have been observed and have caused crosses to fail. Embryo culture is a useful tool to overcome these barriers.

Although in vitro embryo culture in *Salix* has received little attention, lessons can be learnt from protocols developed in *Salix's* sister genus, *Populus*, in the family Salicaceae. Poplars have received more attention in this area as they have a longer seed maturation period than willows. 3–6 weeks in *Salix* compared to 3–5 months in *P. deltoides* (Stanton & Villar, 1996). Raquin *et al*. (1993) *Populus* protocol was chosen as it was as simple compared to others which use complex media supplemented with growth regulators (Savka *et al*., 1987; Park & Son, 1988) and because it was applicable to a wide array of *Populus* species. It is also a higher throughput technique than other protocols as half capsules, rather than individual embryos are cultured.

Materials and Methods

Flow cytometry

In November 2006 initial ploidy estimates of 220 genotypes were made using flow cytometry. This included cultivars, breeding progeny and key species. 20 cm cuttings were collected from the field trials at Rothamsted Research (51º49' N, 0º21' W). Cuttings were reared in water culture; two thirds of the cuttings were submerged. Following bud burst the leaves were aloud to expand. Cuttings were transported to Aberystwyth and leaves were excised as needed to ensure fresh leaf material was available for each run. A plastic Petri dish was prepared containing 2 mL of ice-cold Buffer A (0.5 M citric acid monohydrate, 0.5% Tween 20), 2 cm long pieces of leaf were added and chopped with a razor blade in the vertical position, this released the cell nuclei. The suspension of cell nuclei was then stained by adding 0.5 mL of Buffer B ((0.4 M anhydrous Na_2HPO_4) containing 4 µg mL^{-1} DAPI (4–6-diamidino-2-phenylindole)). Buffer B was kept in the dark when not in use. The Petri dish was then swirled and filtered through a 50 µm nylon mesh and run on the Partec Ploidy Analyser (http://www.partec.com). Histogram peaks of relative DNA content were recorded. Diploid *S. viminalis* 'Bowles Hybrid' and hexaploid *S. dasyclados* 'Korso'

were run as standards.

Where peaks were unclear leaf samples were repeated by Plant Cytometry Services (http://www.PlantCytometry.nl) for ploidy analysis. These were carried out with a Partec CyFlow flow cytometer. The nuclei were released and stained in a similar way to above and were analysed together with an internal standard *Lactuca sativa* L. 'Capitata' (Iceberg lettuce).

Embryo culture techniques

A cross between *S. viminalis* 'Bowles Hybrid' × *S. rossica* was chosen for this initial study as it provides an abundant source of embryos. The cross does not suffer from premature flowers abscission, but to mimic it, the capsules were removed for culture at 3, 4, 9 and 11 days post pollination. The breeding technique used was similar to that described by previous willow breeders (Mosseler, 1989; Lindegaard & Barker, 1997). Fertilised catkins were removed at the desired days post pollination. Beneath a flow hood, and sterilising instruments frequently, any remaining stigma parts were removed, catkins were then surface sterilised for 3 minutes in a solution of calcium hypochlorite at a concentration of 15 g L^{-1} and rinsed twice with sterile distilled water (3 minutes per rinse). Capsules were removed from the catkin, cut longitudinally into halves and embedded in to the media in an upright position at a density of 12 half capsules per 55 × 15 mm Petri dish. Petri dishes were sealed with nescofilm and incubated at 26°C in darkness for 7 days, then moved to a growth compartment with a 16-h photoperiod. Plantlets were transferred to multi-celled trays containing Rothamsted standard compost mix at 4–6 leaf stage. A propagator lid was kept over the plantlets until they were hardened off.

Table 1. *Components of the media trailed compared Raquin* et al*. (1993) media*

(Raquin *et al*., 1993)	MSMO	M5519	WM
½ conc. M&S inorganic salts	½ conc. M&S basal salt with minimal organics	½ conc. M&S basal medium	M&S macrosalts L7 microsalts M&S vitamins (-glycine) Myo-inositol 3AA amino acids 800–1100 M OSAH
Fe EDTA 10 -4 M (Ferrous sulphate chelate solution)	Fe EDTA 10 -4 M	Fe EDTA 10 -4 M	Fe EDTA
PH 5.8	pH 5.8	pH 5.8	pH 5.7
0.17 M Sucrose	0.17 M Sucrose	0.17 M Sucrose	0.26 M Sucrose
6 g L^{-1} agrose	10g L^{-1} agargel	10g L^{-1} agargel	10 g L^{-1} agargel
	PPM 1 mL L^{-1}	PPM 1 mL L^{-1}	PPM 1 mL L^{-1}

Wheat medium (WM) was used as it was readily available from the Cereal Transformation Group at Rothamsted Research. Murashige and Skoog basal salt with minimal organics (MSMO) and Murashige and Skoog basal medium (M5519) powders were purchased from Sigma-Aldrich and formed the basis for the other two media. The broad-spectrum biocide/fungicide for plant tissue culture, Plant Preservative Mixture (PPM) (http://www.ppm4plant-tc.com) was added to all media before the plates were poured. Media components are detailed in Table 1. Successfully germinated embryos were subcultured in to a second media, poured and prepared in the same way as the first three cultures but containing 0.06 M of sucrose.

Results

Flow cytometry

Average crossing success improved from 37.5% in 2006 to 48.7% and 56.4% for 2007 and 2008 respectively. Ploidy estimates being used when planning the crosses were a huge factor in these improvements. Viewing the 2004–2006 crosses retrospectively with the benefit of the data presented here, 28% of crosses attempted failed due to inappropriate combinations involving either triploid or pentaploid parents.

Table 2. *Flow cytometric estimates of the nuclear DNA content of* Salix *species*

Species	Relative florescence (i)	DNA ratio (ii)	Ploidy level (x) (iii)	2n (iv)
S. aurita L.	49.1	-	2	38
S. cordata Muhl. 'Farndon'	56.1	-	2	38
S. daphnoides Vill. 'PRAHA'	62.1	-	2	38
S. eriocephala Michx. 'R632'	50	0.19	2	38
S. purpurea L. 'Lancashire Dicks'	59.0	-	2	38
S. schwerinii Wolf 'K3 Hilliers'	50	-	2	38
S. sitchensis Sanson ex Bong.	59.2	-	2	38
S. triandra L. 'Brunette Noire'	54.4	-	2	38
S. udensis Trautv. Et Mey. 'Sekka'	55.7	-	2	38
S. aegyptiaca L.	96.9	0.36	4	76
S. alba L. 'Gontrode Aalmoeseneie 81004'	97.5	-	4	76
S. babylonica L.	113.8	-	4	76
S. caprea L.	105.0	0.36	4	76
S. fragilis L. 'R838'	102.3	-	4	76
S. miyabeana Seemen 'Purpurescens'	96.5	0.32	4	76
S. miyabeana Seemen 'Shrubby'	102.5	-	4	76
S. pentandra L. 'MacMillan Bloedel'	94.6	-	4	76
S. rehderiana Schneider	110.8	-	4	76
S. scouleriana Barratt ex Hook. 'B PN 215'	97.3	-	4	76
S. rigida 'Mackenziana'	148.7	0.51	6	114
S. hookeriana Barratt ex Hook.	135.2	-	6	114

(i) Relative florescence of cytometric peak measured on the PA at Aberystwyth
(ii) DNA ratio with internal standard *Lactuca sativa* 'Capitata' (Iceberg Lettuce) measured by Plant Cytometry Services
(iii) Estimated ploidy level
(iv) Estimated chromosome number (2n).

Relative florescence of cytometric peaks for diploid, triploid, tetraploid, pentaploid and hexaploid individuals ranged from 40.5–62.10, 69.82–74.07, 94.64–113.81, 116.22–118.66 and 117.01–148.72 respectively. Results of relative DNA ratio in relation to the standard *Lactuca sativa* 'Capitata', measured by Plant Cytometry Services, showed much less variation, diploid, triploid, tetraploid, pentaploid and hexaploid individuals ranged from 0.17–0.19, 0.24–0.25, 0.32–0.36, 0.42 and 0.48–0.51 respectively.

Table 3. *Flow cytometric estimates of the nuclear DNA content of biomass hybrids*

Species	Relative florescence (i)	DNA ratio (ii)	Ploidy level (x) (iii)	2n (iv)
((S. viminalis × S. viminalis) × (S. viminalis × S. schwerinii)) × (S. viminalis × (S. viminalis × S. schwerinii)) 'Resolution'	48	0.17	2	38
S. schwerinii × (S. viminalis × S. schwerinii) 'Discovery'	-	0.17	2	38
S. schwerinii × (S. viminalis × S. viminalis) 'Endeavour'	50	-	2	38
S. viminalis × (S. viminalis × S. schwerinii) 'Olof'	47.7	-	2	38
S. viminalis × (S. viminalis × S. schwerinii) 'Quest'	46.1	-	2	38
S. viminalis × (S. viminalis × S. schwerinii) 'Sven'	47.7	-	2	38
(S. schwerinii × S. viminalis) × S. viminalis 'Tordis'	51.8	0.17	2	38
(S. schwerinii × S. viminalis) × S. viminalis 'Torhild'	50.1	0.17	2	38
S. viminalis × S. schwerinii 'Bjorn'	50.4	-	2	38
S. viminalis × S. schwerinii 'Tora'	40.5	0.18	2	38
S. viminalis 'Bowles Hybrid'	50	0.18	2	38
(S. viminalis × S. triandra) × S. miyabeana 'Terra Nova'	74.1	0.25	3	57
(S. viminalis × S. schwerinii) × S. miyabeana 'Nimrod'	69.8	0.25	3	57
S. rehderiana × S. dasyclados 'Endurance'	116.2	0.42	5	95
S. dasyclados × S. viminalis 'Stott 10'	118.7	0.42	5	95
S. dasyclados 'Aud'	117.0	0.48	6	114
S. dasyclados 'Korso'	135.6	0.49	6	114
S. dasyclados 'Loden'	139.7	0.49	6	114

Table 4. *Flow cytometric estimates of the nuclear DNA content of breeding progeny*

Species	Relative florescence (i)	DNA ratio (ii)	Ploidy level (x) (iii)	2n (iv)
(S. viminalis × S. schwerinii) (2×) × *S. miyabeana* (4×)	-	0.24	3	57
(S. viminalis × S. schwerinii) (2×) × *S. dasyclados* (6×)	-	0.33	4	76
S. rigida (6×) × *S. miyabeana* (4×)	-	0.42	5	95

Embryo culture techniques

Healthy seedlings were obtained from embryos rescued 8, 10 and 11 days post pollination. Initial growth was seen in embryos as young as 3 days post pollination but plantlets developed poorly, eventually dying. Across all treatments an average of 5–9 embryos developed per half capsule. MSMO and M5519 out performed the WM. Before a contamination event, M5519 was averaging 7.7 plantlets per half capsule and MSMO 6.5 plantlets per half capsule. The contamination of some media with mould was caused by the PPM being accidentally omitted from a batch of cultures. Despite this the protocol proved robust at 8 days post pollination, with embryos yielding an averaged 5.8 plantlets per half capsule and more successful at 10 days post pollination embryos

averaged 8.0 plantlets per half capsule. Despite M5519 cultures yielding more plantlets per half capsule than plantlets from the MSMO media, MSMO plantlets transferred to peat plugs with a 68% success rate compared to a success rate of only 23% for M5519 seedlings.

Other techniques to overcome crossing barriers

Phased removal of well wrapped breeding rods from cold storage (-4°C) has overcome many of the problems associated with non-synchrony of flowering. Paying attention to the flowering nature of the parental species is also important. Special care was found to be important when interspecific crosses involved serotinous or coetaneous species were crossed with a precocious flowering habit species. In intraspecific crosses female parents were found to flower 1–2 days earlier than males. Delayed removal of females from cold storage improved synchronisation and crossing success. Synchronization of male and female catkins was occasionally not possible. This was overcome by using Kopp *et al*. (2002) pollen collection and storage protocol which involves extracting pollen using toluene.

A small number of crosses where pistillate parents have failed when cut stems are used in water culture have been repeated successfully with potted rooted material. Examples include *S. rehderiana, S. dasyclados* and *S. eriocephala*.

Discussion

Flow cytometry

Flow cytometry has proved an important tool in improving the efficiency of the BEGIN programme. It has also had the additional benefit of allowing other crossing barriers to be focused on as failure caused by inappropriate ploidy combinations can be discounted.

Some limitations with the methodology used should be noted. Absolute DNA amounts were not obtained as DAPI, the fluorescent dye used, has specific DNA-binding properties with prefer adenine-thymine (AT) rich sequences. As the AT/CG ratio of plants can not be assumed as 1:1, DAPI is not suited for measurements of absolute DNA amount. Because of this our results can not be compared directly to Thibaut (1998). Due to time constrains each of the 220 genotypes screened at Aberystwyth were only run once. Average readings of repeated runs should have been taken in order to account for variation caused by the high pressure mercury lamp heating up. This may explain the variation in the results. The technique should generally offer reliable estimation of ploidy levels, but differences in DNA amount between species may vary, making estimation ploidy levels in some instances unreliable.

Table 4 shows the ploidy levels of progeny of hybrids produced in the BEGIN crossing programme, they display the intermediate ploidy level in comparison to their parents. Ten siblings from each cross were screened per cross and their relative DNA amounts were the same. Care should be taken though as variety 'Stott 10' is a pentaploid. This ploidy level is not the intermediate value of the two parents and could be a result of the spontaneous mechanism of aneuploidy. Bradshaw & Stettler (1993) found that tri/aneuploid progeny from a (2×) × (2×) interspecific cross were surprisingly common with 10 out of 15 crosses producing tri/aneuploid progeny, several in high proportions. Predicted ploidy levels should only therefore be used tentatively when planning advanced generation breeding schemes as additional screening will be needed to identify any aneuploids.

Embryo culture techniques

The embryo culture protocol developed can now be used to rescue embryos from catkins that would otherwise fail. Lessons learnt when developing the protocol such as the importance of operating in a sterile environment were vital. Further work is still needed in improving success rates when transferring plantlets to the glasshouse. Despite this, the protocol will be used to attempt to overcome crossing barriers and will prove especially useful in overcoming crossing barriers between subgenera and sections. Notoriously difficult crosses between subgenera Vetrix and *Salix* good targets for this protocol.

Other techniques to overcome crossing barriers

Another method of overcoming crossing barriers can be learnt from Populus breeders. Crossing failures from cut stem material have been overcome using potted rooted material instead (Stanton & Villar, 1996). Success of previously failed crosses, repeated using potted rooted female material, is likely to be due to a functional root system providing higher moisture and nutrient levels to the developing seeds.

Acknowledgments

BEGIN (http://www.biomass4energy.org) is funded by the Department for Environment, Food and Rural Affairs project No. NF0424. We are also grateful to C Sparks and Dr H Jones of the Cereal Transformation Group at Rothamsted Research for their advice and help in developing the embryo culture technique and finally to Dr G. Geenen at Plant Cytometry Services for providing an excellent service. Rothamsted Research receives grant-aided support from the BBSRC.

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