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Technique Development Towards Production of an *In Vitro* Graft Chimera in *Rubus*

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Abstract

In vitro grafting was attempted to produce periclinal chimeras in *Rubus*. The effect of plant growth regulators on the preparation of graftable material, *in vitro* grafting and regeneration of shoots from graft union tissue was investigated on two thornless and two thorny blackberries. Grafted plants grown on medium containing no plant growth regulators, 1 μM abscisic acid or 10 μM paclobutrazol produced 79 to 84% successful grafts compared to 20 to 50% successful grafts on medium containing Ethrel or indolebutryic acid. Percentage shoot formation from excised graft unions ranged between 19 and 40%, only 5 μM indole butyric acid significantly reduced regeneration from the more organogenic treatments. Regenerant shoots were primarily from the scion in ethrel, no plant growth regulator and paclobutrazol treated graft unions. Other treatments resulted in equal numbers of shoots with scion and rootstock phenotypes. Although over 200 regenerants were obtained from both sides of the graft union, no thornless/thorny chimeras were recovered from the subsequent use of this technique.

Introduction

When a particular pest is limited to the epidermis, for example, powdery mildew *(Unicinula necator)* of *Vitis* (6) and powdery mildew *(Sphaerotheca macularis)* of strawberry *(Fragaria)* (11), and apple *(Malus)* Scab *(Venturia inaequalis)* (24), producing a periclinal chimera with a pest resistant epidermis may be an effective method to improve pest resistance and maintain cultivar performance in asexually propagated plants. Several periclinal solanacous species chimeras have been synthesized and have resulted in increased stress tolerance or pest resistances (4, 13, 14). These chimeras were produced in the greenhouse by grafting two dissimilar genotypes and regenerating adventitious shoots from the graft union. The process has not been adapted to woody species because shoot regeneration from internodal graft union tissue has not been obtained *in vivo*. Recent advances have produced techniques for this type of regeneration from a wide range of species *in vitro* (28).

The *in vitro* production of graft chimeras has not been reported. The formation of thornless blackberry graft chimeras *in vitro* are theoretically possible since thorniness in *Rubus* is determined by epidermal (L-1 histogen) genotype and chimeral thornless blackberries exist, eg. *R. laciniatus* 'Evergreen Thornless' (5). Therefore, experiments were designed to develop a protocol of *in vitro* techniques that mimic graft chimera formation *in vivo*.

Material and Methods

A set of sequential experiments were conducted on the effect of plant growth regulators on shoot elongation, graft union formation and healing, shoot organogenesis from graft union tissues, and regenerated shoot genotype.

Plant Materials

Rubus sp. subgenera *Eubatus* 'Austin Thornless' and 'Loch Ness,' which are genetically and homogeneously thornless, were the scions and potential epidermal donors. The thorny ETCE-1 ('Black Satin' x 'Tayberry') and 'Shawnee' blackberries were the rootstocks. Various phenotype markers were available. Compared to other cultivars, 'Austin Thornless' has a much

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wider leaf with coarser serrations; 'Shawnee' is an upright plant; ETCE-1 and 'Loch Ness' are semi-erect and 'Austin Thornless' has a prostrate growth habit.

Media and culture conditions

The *in vitro* plant material used for this study was initiated and maintained in cultures containing Murashige and Skoog (MS) basal (18) medium supplemented with 7 μM benzyladenine (BA), 1 μM indolebutyric acid (IBA) and 0.25 mM adenine sulfate. All plant material was grown at 20° ± 3° C under continuous 30 μmol s^{-1} m^{-1} cool white fluorescent lamps and subcultured every four to six weeks.

For all experiments, the basal medium was Murashige and Skoog inorganic salts and vitamins (18) supplemented with 90 mM sucrose, 0.25 mM adenine sulfate, pH adjusted to 5.7 before 15 min autoclaving, and solidified with 7 g l^{-1} Difco Bacto agar. Plant growth regulators (PGRs) were added to MS medium as described in each experiment. Ethrel [(2-chloroethyl) phosphonic acid] (an ethylene-releasing agent), gibberellin A_3 (GA), kinetin and paclobutrazol (PP333) were filter-sterilized using 0.22 μm disposable syringe-fitting filters. Thidiazuron (TDZ) was dissolved in acetone and added after autoclaving but before the medium cooled to less than at 90°C. Abscisic acid (ABA), BA, 2,4-dichlorophenoxyacetic acid (2,4-D), IBA and naphthaleneacetic acid (NAA) were added to MS media before autoclaving.

General methodology

Three general procedures were studied: 1) preparation of elongated shoots for grafting, 2) grafting and graft union healing, and 3) graft union shoot organogenesis.

1) Several experiments were compared the effects of PGRs on stem elongation, thickness and rooting. Because only adventitious buds developing at the graft union are potential graft chimeras, the use of elongated internodes is desirable to reduce inclusion of axillary buds in the subsequent graft union explant.

The following protocol was used for all experiments, except where indicated. For each replication, the top 1 cm. of a one month old *in vitro* shoot was placed in a capped and parafilm wrapped glass test tube (25 x 150 mm) containing 10 ml of medium. Various PGR treatments and the number of replicate explants per treatment are specified in Table 1. Stem height, internode length and thickness, and numbers of roots and shoots per explant were measured after two months of culture.

2) *Rubus* plantlets from MS medium containing 2.5 μM NAA were elongated and rooted and thus used for this experiment. Since cell division is necessary before vascular differentiation (7) and graft union healing has been aided by cytokinins (12), the rootstocks and scions were submerged in 10 ml MS liquid medium with 5 μM zeatin for two to three hours before grafting. Preliminary experiments indicated this increased graft healing from 13% to 42% in *Malus* (data not shown).

Grafting was performed under a dissecting microscope (Zeiss SV8) with 8X magnification. All leaves were excised, except the two apical leaves of the scions. The rootstock was decapitated at the longest internode and a basal-pointing 1-3 mm long wedge shaped cut was made. A reverse wedge-shaped cut was made at an internode approximately 1 cm from the apex of the scion shoot (Figure 1). The scion was carefully inserted into the V-shaped groove of rootstock and wrapped with a 3 to 5 mm length of autoclave sterilized silicone rubber tubing (id. 1 mm, od. 3 mm). A slightly sigmoid-shaped cut was previously made down the length of the silicone rubber tubing to allow application and expansion of the grafting rubber. Micrografts were placed in 100 ml

volume (10 cm tall) glass bottles containing 35 ml of MS medium with the treatments indicated in Table 2. In the first month, cultures were grown under continuous dim light (5 μmol s^{-1} m^{-1}); in the second month, cultures were moved to the standard environment.

After two months of culture, the percentage of grafting success was determined under a dissecting microscope. Silicone rubber tubing was first removed with forceps. Healed grafts had a small amount of callus cells proliferated on the cut surfaces of scion and rootstock, and the graft union did not break upon excision during organogenesis studies. Unhealed graft unions could be easily separated by forceps and always broke under the slightest pressure of a scalpel blade.

3) Graft unions of the *Rubus* micrografts generated in the previous subculture were used to conduct this section of the experiment. To excise the graft union, three cuts were made at the micrograft. Working under the dissecting microscope, one cut was made across the rootstock 1 mm below the base of the graft union, another cut was made on the scion 1 mm above the graft union, and the final cut was made diagonally along the graft union (Figure 1). This procedure produced a rootstock internode which contained a 1 mm thick section of scion tissue. The scion section of the excised graft union was placed face-down on medium previously used for shoot organogenesis from *Rubus* internodes (8, 9). This organogenesis medium contained MS salts, vitamins, Staba vitamins (21), and 5 μM thidiazuron (TDZ) (8, 9). Because treatment PGRs were not reapplied in this subculture, any effects were from the previous subculture. After a week in the dark, cultures were placed in the standard environment.

Adventitious shoots regenerated from the graft unions, these shoots were transferred to MS medium containing 0.6 μM zeatin to stimulate shoot elongation without proliferation. After two months of culture, the number of graft unions that regenerated at least one shoot were counted and the genotype was determined by phenotypic markers, i.e. thorniness and leaf shape.

Random amplified polymorphic DNA (RAPD) analysis

Leaf DNAs were isolated from genotypes 'Austin Thornless,' 'Loch Ness' and thorny 'ETCE' and two putative graft chimeras. A modified CTAB (hexadecyl trimethyl ammonium bromide) procedure (20) was used without the final PEG precipitation step.

Amplification reactions were performed in volumes of 25 μl using a procedure described in Levi et al., (15). The reagents and conditions included 50 mM Tris HCl-pH 9.0, 20 mM NaCl, 1.6 mM $MgCl_2$, 1% Triton X-100, 0.1% Gelatin, 0.2 μM primers, 200 μM of each dNTPs, 0.028 units/μl of Taq DNA polymerase (Promega Corporation, Madison, WI) and 1 mg/ml of template DNA. Oligonucle-

Table 1. The effect of naphthalene acetic acid and thidiazuron on the internode length in mm of the shoots of various *Rubus* sp. genotypes.

Treatment	Austin TL	ETCE-1	Loch Ness	Shawnee
no PGR	2.1b	4.2bc	5.4ab	1.9c
2.5 μM NAA	4.1a	5.8ab	7.2a	5.5a
100 nM TDZ	-[hb]	5.7ab	3.2bc	2.7bc
250 nM TDZ	-[hb]	5.0abc	2.5c	4.4ab
2.5NAA+100TDZ	4.8a	6.8a	3.3bc	6.0a
2.5NAA+250TDZ	-[hb]	3.3c	-[hb]	-[hb]

[hb]—explants highly branched—no internode length data taken.
Least Significant Differences at 5% for means of each genotype are indicated by different letters.

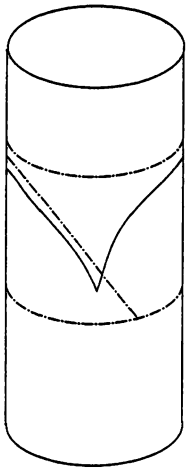


Figure 1. A representation of a section of a grafted shoot. The V indicates the graft union. Dashed lines indicate the excision cuts used for regeneration of shoots.

otide primers were synthesized by The Biotechnology Laboratory, University of British Columbia. Primers with a GC content of >70% were used (Figure 2).

DNA was amplified in a MJ programmable thermal control (Model PTC-100, MJ Research, Watertown, Mass.) programmed for 45 cycles of 40 sec at 94°C, 1 min at 48°C and 2 min at 72°C. Amplification products were analyzed by electrophoresis in 1.4% Sigma agarose gels and detected by staining with ethidium bromide.

Statistical analysis

Data were analyzed by analysis of variance (SAS Procedure GLM) with significant effect means separated by LSD or Duncan's multiple range test at the 5% probability level.

Results

Elongation Experiments

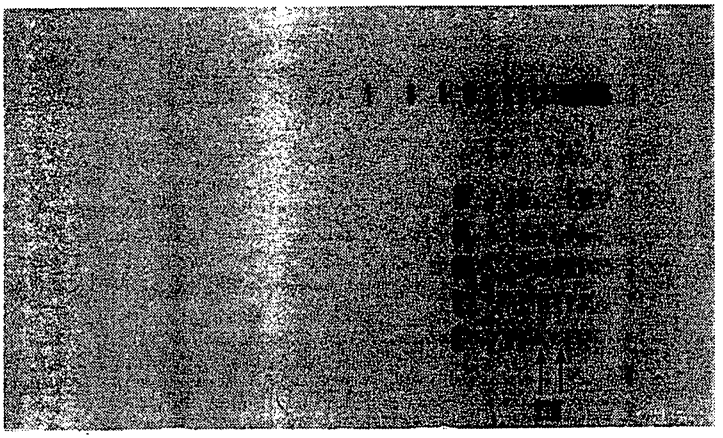
In two preliminary experiments (data not shown), 1.5 and 3 μM GA, 2.5 and 5 μM TDZ and 2.5 μM NAA were added to the media to facilitate manipulation of internodal grafting sites. GA-treated explants elongated more than controls devoid of PGRs; however, shoots were thinner than other treatments and controls. Most of the TDZ-treated shoots were thicker than controls; however, internodes were shorter than other treatments. NAA-treated explants had both thick and elongated internodes. For all four genotypes, 2.5 μM NAA-treated explants also produced the most number of roots per explant.

In the first comprehensive experiment, 2.5 μM NAA-treated explants were unbranched and had longer internodes and greater explant height than control plants in all genotypes ($p < 0.05$) except 'Loch Ness' and ETCE-1, where only the total explant height was significantly greater than controls (Table 1). NAA-treated internodes (1.7 ± 0.25 mm avg.) were significantly ($p < 0.05$) thicker than controls (1.0 ± 0.1 mm avg.) for all genotypes except 'Austin Thornless,' which had 1.0 mm thick internodes regardless of treatment. TDZ effects on internode elongation were inconsistent, or in the case of 'Loch Ness,' contrary to the desired result. In addition, TDZ did not improve internode thickness (data not shown). Thus, TDZ was not used for the explant elongation and thickening step.

In the second experiment (data not shown), two concentrations of various auxins and cytokinins were added to the shoot elongation stage medium. Few differences were found between the two levels of each plant growth regulator and the results were pooled. As in previous experiments with TDZ, the cytokinins, kinetin and BA, reduced internode elongation and did not improve thickening. Compared to NAA, the auxins IBA and 2,4-D did not improve internode elongation or thickening at either of the concentrations tested.

Graft Union Healing

In a series of preliminary experiments, silicon rubber tubing was re-



Primers and Primer Sequences used for RAPD analysis.

Primers	Sequences	GC Content
P_1	ACC GTG GGT C	70%
P_2	CGG GGC GGA A	80%
P_3	GGG GGC CTC A	80%
P_4	AGC GCC GAC G	80%
P_5	GGT CGC AGC T	70%
P_6	TGC GCA GCC C	80%

Figure 2. Amplification of Austin Thornless (A), ETCE (E) and putative graft chimera (C) DNAs using the indicated primers (P_1 to P_6—see above). RAPD fragments generated from parent plants (A and E) are compared with that of chimera (C). Note, for ease of viewing, the image presented was the computer-generated negative of the ethidium bromide-agarose gel.

quired for union healing: vertically oriented grafted explants healed at a higher success rate (38%: n = 44) than those placed horizontally on the medium (24%: n = 34). Horizontally placed explants were excessively callused obscuring the location of the graft union.

The effect of PGRs was tested on healing and subsequent shoot regeneration of graft genotypes. Genotype effects were insignificant, therefore data were summarized by PGR treatments (Table 2). In general, graft union healing was complete after 2 months for most control-treatment explants (Table 2). ABA- and PP333- treated explants also healed at a high rate; however, the apical region of these plants did not elongate as in controls. Ethrel and IBA treatments reduced graft healing. These growth regulators resulted in the formation of non-morphogenic (friable) callus at all cut surfaces.

Shoot regeneration

Except for 5 μM IBA, plant growth regulators did not alter shoot regeneration from excised graft union pieces (Table 2). Again, IBA-treated explants were excessively callused and the organogenesis medium only stimulated further friable callus development. All excised 5 μM IBA-treated graft unions were subsequently placed on medium containing 7 μM BA; none regenerated. Genotype effects on regeneration were insignificant.

Regenerated plant phenotypes

The effect of PGR treatment, applied at the graft union healing step, on the proportion of shoots regenerating from either the scion or root stock genotype was substantial, but was not correlated to graft union or organogenic behavior (Table 2). The eight shoots obtained from ethrel-treated

Table 2. The effect of plant growth regulators, applied during the graft union healing step, on the percentage graft union healing, the percentage of excised graft union explants that regenerated shoots and the proportion of regenerated shoots from the scion vs. the rootstock genotype used.

Graft Treatment	% Successful Graft Unions	Organogenesis %	Scion/Rootstock proportion	No. scion shoots regenerated	No. rootstock shoots regenerated
Control	82	19	18:1	74	4
1 μM ABA	84	27	1:1	19	16
30 μM Ethrel	20	22	all scion	8	0
3 μM GA	55	25	1:1	14	16
0.5 μM IBA	50	22	1:1	18	14
5 μM IBA	41	0	--	--	--
10 μ Paclo	79	40	3:1	20	7
LSD (0.05)	44	27	--		
Average Number of Observations	61	24	27		

explants were from the scion genotype. Seventy-four of the 78 shoots from control-treated internodes were also scion genotypes. ABA-, IBA- and GA-treated regenerants produced nearly equal amounts of scion and rootstock genotypes.

Two off-type plants with a lower number of thorns and moderately puckered leaves were obtained from the 208 plants regenerated in these experiments. One was attached to an ETCE-1 phenotype shoot from an 'Austin Thornless'/ETCE-1 graft union. The other off-type was found on a 'Loch Ness'/ETCE-1 plant that had both parental phenotypes and the off-type. After a year in the field, only the ETCE-1 phenotype was found.

RAPD analysis was used to confirm whether the off-type regenerants were true chimeras (Figure 2). The off-type from the 'Austin Thornless'/'ETCE-1' graft appears to be related to ETCE-1. 'Austin Thornless'-specific markers are absent in the offtype. Unlike ETCE-1, however, the AT/ETCE-1 offtype has an extra band at 500 bp and a missing band at 1300 when primer 6 was used (indicated by arrows), indicating it is probably a mutation of ETCE-1. The 'Loch Ness'/ETCE-1 off type had markers identical to ETCE-1 (data not shown).

An artificial pre-amplification DNA mixture of 1 part 'Austin Thornless' to either 9, 29 or 49 parts ETCE-1 was used to determine the sensitivity of the RAPD procedure used. In this test, the amount of DNA per sample was 25 ng. As noted by the presence of bands at 1100 and 1300 bp (indicated by arrows), 'Austin Thornless' could be detected in as little as one part in 30 of the total sample DNA (Figure 3).

Discussion

No graft chimeras were recovered from the regenerants obtained in these experiments; however, techniques were developed which mimic the graft union techniques which have produce chimera in herbaceous plants (4, 26; as reviewed by 23). In those *in vivo* experiments, the rate of chimera production ranged from none to greater than one in a hundred. Thus, there may have been sufficient numbers of regenerants obtained to allow a reasonable chance for formation of chimeras.

Shoot regeneration from *in vitro* mixed cell suspensions have also been used to attempt to regenerate chimeral

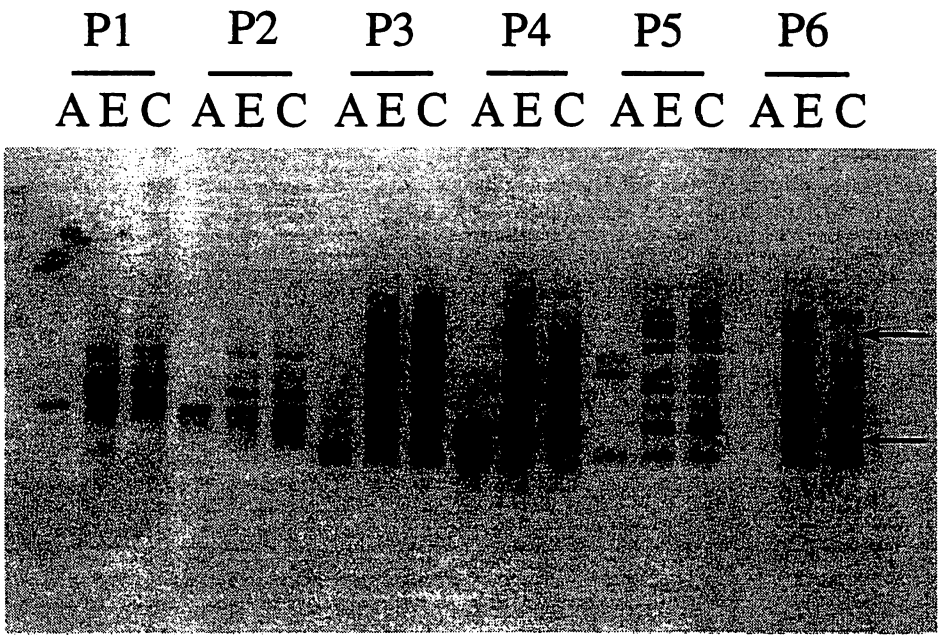


Figure 3. Sensitivity of the RAPD technique as determined by mixing 'Austin Thornless' and ETCE-1 DNA in ratios of 1:9, 1:29 and 1:49 by weight. 'Austin Thornless' (AT) and ETCE-1 and ETCE-1 off-type (Ch) patterns are also presented. The arrows indicate diluted 'Austin Thornless' bands at approxiamtely 1000 and 1200 bp in the mixture. The lane on the far left refers to the 100 bp ladder with a double concentration of the 600 bp fragment (Gibco BRL, Gaithersburg, MD). Primer 5 (Figure 2) was used; empty lanes were test mixture controls. Note, for ease of viewing, the image presented was the computer-generated negative of the ethidium bromide-agarose gel.

plants (1, 2, 3, 16). These attempts have resulted in unsuccessful or lower rates (ca. 0.1%) of chimera formation. Coordinated cellular development may be necessary for chimeral shoot formation. Vascularization across the union was visable in the second month of culture, indicating the graft partner cells were coordinating cell development to some degree at the time of graft union excision and shoot organogenesis.

Formation of chimeras may be less likely under *in vitro* conditions. Tissue culture explants commonly exhibit a rejuvenated phenotype which can be reversed to a degree by the application of ABA (22). In this study, ABA did not change the rates of graft union healing or organogenesis and ABA-treated graft unions produced plant equally from both graft partners. Thus, the inclusion of ABA to the graft medium is recommended because the condition of the shoots may more closely approximate those grown *in vivo*.

IBA and ethrel inhibited graft vascularization and knitting, which was inconsistent with previous *in vivo* results (10, 19, 25, 27). Graft unions treated with IBA and ethrel had friable callus, a state closely related to mixed callus than to well-healed *in vivo* graft unions. In addition, regeneration from IBA-treated explants was reduced.

For the above reasons, *in vitro* graft chimera formation in woody plants is probably less frequent than *in vivo* formation. It is possible sectoral or periclinal chimeras may have formed, but one genotype may have displaced

the other due to uneven growth rates. However, all genotypes used in these experiments, except 'Austin Thornless,' grew at relatively the same rates. In addition, a high percentage of the regenerated shoots were from the scion genotype, even though the scion piece was relatively small (less than 10% of the explant volume). The small amount of zeatin applied to grow the small regenerant shoots to rooting size did not result in axillary branching. Rapidly branching strawberry chimera were lost when grown on cytokinins *in vitro* (17).

The rates of graft union formation here were double those reported in studies with meristem-tips (12). The rate of shoot regeneration from graft unions was slightly less than that reported for internodes with TDZ (9). Because TDZ (5 μM) was reported previously as more effective than BA for internode regeneration in *Rubus*, other cytokinins will not likely enhance regeneration from graft unions (9).

Because *in vitro* shoot regeneration and grafting are successful for many woody plants (28), *in vitro* graft chimeras of other species which do not readily form adventitious shoots *in vivo* may be possible. Future manipulation of regeneration from the two graft partners may be improved by inclusion of kanamycin resistance and cytokinin production genes in either of the genotypes. When phenotypic markers are lacking, identification of graft chimera may also be aided by histological staining of β-glucuronidase transformed plants.

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