Micrografting techniques for testing long-distance signalling in Arabidopsis

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Summary

Grafting in species other than Arabidopsis has generated persuasive evidence for long-distance signals involved in many plant processes, including regulation of flowering time and shoot branching. Hitherto, such approaches in Arabidopsis have been hampered by the lack of suitable grafting techniques. Here, a range of micrografting methods for young Arabidopsis seedlings are described. The simplest configuration was a single-hypocotyl graft, constructed with or without a supporting collar, allowing tests of root–shoot communication. More complex two-shoot grafts were also constructed, enabling tests of shoot–shoot communication. Integrity of grafts and absence of adventitious roots on scions were assessed using plants constitutively expressing a GUS gene as one graft partner. Using the max1 (more axillary growth) and max3 increased branching mutants, it was shown that a wild-type (WT) rootstock was able to inhibit rosette branching of mutant shoots. In two-shoot grafts with max1 and WT shoots on a max1 rootstock, the mutant shoot branched profusely, but the WT one did not. In two-shoot grafts with max1 and WT shoots on a WT rootstock, neither shoot exhibited increased branching. The results mirror those previously demonstrated in equivalent grafting experiments with the ramosus mutants in pea, and are consistent with the concept that a branching signal is capable of moving from root to shoot, but not from shoot to shoot. These grafting procedures will be valuable for revealing genes associated with many other long-distance signalling pathways, including flowering, systemic resistance and abiotic stress responses.

Keywords: Arabidopsis thaliana, max mutants, grafting, shoot branching, long-distance signal.

Introduction

Many lines of research covering several decades support the concept that plants use a wide range of long-distance or systemic signals. For example, genetic and physiological evidence indicates that roots subjected to various stresses alter the export of specific compounds, such as ACC and ABA, to the shoot via the xylem stream (Bradford and Yang, 1980; Schurr et al., 1992), leading to responses such as altered stomatal aperture and leaf growth (Thompson et al., 1997). Classic grafting experiments demonstrate photoperiodic regulation of long-distance signalling from leaf to shoot apex that influences transition to flowering (e.g. Chailakhyan, 1968; Lang et al., 1977). However, conclusive identification of the nature of the transmitted floral stimuli and/or inhibitors has remained frustratingly elusive. One problem is that, with the exception of pea (Pisum sativum), much of the work has been conducted in species that are not ideal genetic models. In pea, however, there are several mutants that affect graft transmission of a floral inhibitor, and at least one that affects a floral stimulus (Beveridge and Murfet, 1996; Murfet, 1971; Weller et al., 1997). Despite an abundance of mutants in Arabidopsis affecting processes such as flowering, and substantial information on corresponding genes and gene products, it remains difficult to predict Arabidopsis homologues of the genes from other species. With respect to control of flowering, direct comparison between Arabidopsis and pea is further
complicated by apparent differences in regulation between the two species, in that at least four pathways have been proposed for *Arabidopsis*, but essentially only two in pea (Haughn et al., 1995; Weller et al., 1997).

A major impediment has been the lack of suitable grafting techniques in *Arabidopsis*. Two previous papers describe grafting of the inflorescence stem of 30-day-old plants (Rhee and Somerville, 1995; Tsukaya et al., 1993). However, at this stage most critical developmental events (such as floral induction and shoot branching) have long since passed. Grafting of *Arabidopsis* seedlings would provide unlimited options for combining different shoot and root genotypes. Outcomes in terms of phenotype and alterations in gene expression are likely to give many clues as to gene functions and the nature of the transmitted signals.

One system where grafting has led to new models for long-distance signalling is in the regulation of shoot branching. Mutations that cause an increase in bud outgrowth compared to wild-type (WT) have been identified in five *RMS* (*Ramosus*) loci in pea (Beveridge, 2000; Napoli et al., 1999). Grafting experiments have revealed that three of the mutants (*rms1*, *rms2* and *rms9*) exhibit near-WT bud outgrowth if grafted onto a WT rootstock (Beveridge et al., 1994; Beveridge et al., 1997b; Morris et al., 2001). This led to the hypothesis that the mutants lack a long-distance signal that regulates branching. The amount of tissue required to restore branching to the WT is small, as a short WT epicotyl interstock is as effective as an entire rootstock (Foo et al., 2001). Similar branching mutants have been isolated in petunia, designated *dad* (*decreased apical dominance*; Napoli, 1996; Napoli and Ruehle, 1996). In *dad1*, grafting can lead to restoration of branching from mutant to WT. However, development of adventitious roots on the scion blocks this effect (Napoli, 1996), a phenomenon not reported in pea. The use of Y-shaped grafts with WT and mutant shoots on a mutant rootstock has further demonstrated that, in pea, the branching signal appears to move only acropetally in shoots (Foo et al., 2001). Two other *ramosus* mutants, *rms3* and *rms4*, do not have their branching rescued by grafting, suggesting that they act in a tissue-autonomous manner (Beveridge et al., 1996).

Classic models for regulation of shoot branching invoke two hormonal signals: apically derived auxin and basally derived cytokinin that, respectively, repress and promote bud outgrowth (reviewed by Cline, 1991). Hormone analysis of *rms* mutants indicates that auxin and cytokinin levels are often perturbed. However, the nature of the changes suggests that these two hormones are unlikely to be directly responsible for the graft-transmissible regulation of branching, and thus the existence of at least one novel branching signal has been proposed (Beveridge et al., 1997a; Morris et al., 2001).

A number of mutants that display increased shoot branching have been isolated from *Arabidopsis*. Unlike the *rms* and *dad* mutants, many of these are highly pleiotropic, and some are involved with the biosynthesis/perception of known hormones (Leyser et al., 1996; Lincoln et al., 1990; Talbert et al., 1995). However, one class, the *max* (*more axillary growth*) mutants, exhibit increased axillary branching as the predominant phenotype (Booker et al., 1999; Stirnberg et al., 2002). At present, the sites of action of the *MAX* genes, and correspondence of *MAX* genes to *RMS* and *DAD* genes, have not been reported.

Given the current gaps in knowledge of regulation of many different developmental traits by long-distance signals in *Arabidopsis*, and the importance of such data in drawing comparisons between *Arabidopsis* and other species, we have developed a range of techniques for grafting *Arabidopsis* seedlings. These methods have enabled us to show that *MAX1* and *MAX3* regulate signals capable of acting over long distances to regulate shoot branching.

### Results

#### Development of grafting methods

#### Single grafts

Preliminary experiments indicated that two seedling grafting methods, transverse cut (Figure 1a) and wedge graft (Figure 1b), both gave a satisfactory success rate, with 20–50% of grafted plants growing on to maturity. Improved success rates subsequently achieved are described later. With practice, up to 20 grafts could be assembled per hour. In most experiments, around 50% of grafts formed a good union, with the remainder failing to unite due to imprecise alignment or low seedling vigour. Degree and continuity of tissue contact were major factors in generating rapid and secure unions. A proportion of grafts never formed unions, but this was not dependent on genotype combinations. Optimum age for grafting was 3–4 days on nutrient-free media, and up to 9 days if nutrients were supplied. Scion bending during growth was minimized by orienting plates vertically with lighting from above. Additionally, short lengths of fine-bore silicon tubing were highly effective as a supporting collar placed over the transverse cut grafts. Graft partners that separated rarely formed good unions, even if realigned 24 h after initial grafting.

Up to 50% of grafts developed adventitious roots on the scion. These became visible from 3 to 4 days after grafting and were immediately excised. Sometimes further adventitious roots regenerated, even after excision, in which case grafts were usually discarded. Generally, rapid formation of a graft union reduced the probability of adventitious rooting. In addition, there was an influence of cutting position, with grafts made in the upper region of the hypocotyl being less susceptible than those made in the lower region to...
adventitious rooting (data not shown). Grafts with a collar were slightly more difficult to inspect for early signs of root formation on the scion (Figure 2a), and subsequent removal of the collar sometimes revealed adventitious roots growing through the hypocotyl of the rootstock. With all methods, great care was therefore needed to distinguish grafted plants from adventitiously rooted scion cuttings where the original rootstock had not survived. Verification

Figure 1. Arabidopsis plants 4 days after grafting. Graft types are 90° butt graft (a); wedge graft (b); two-shoot Y-graft (c). Scale bar, 200 μm. Arrow indicates position of graft union in (a).

Figure 2. Grafted Arabidopsis plants later in development. Arrows indicate location of graft union. (a) Butt graft union showing collar on plant 10 days after grafting. Arrowhead indicates adventitious root emerging from base of collar. (b) Plant growing in pot 14 days after grafting. Inset shows detail of butt-type graft union. (c) Mature plant 56 days after grafting. Old leaves around rosette base were excised to facilitate view of graft.

Figure 3. Mature, grafted Arabidopsis plants stained with X-Gluc to visualise β-glucuronidase activity. One partner in each graft carried a CaMV-35S::GUS gene. (a) WT scion on GUS rootstock; (b) GUS scion on WT rootstock; (c) GUS scion in Y-graft onto WT plant; (d) WT scion in Y-graft onto GUS plant. Note absence of adventitious roots on all scions. Leaves were excised and roots trimmed prior to staining.

of graft integrity was most easily achieved using constitutive GUS-expressing plants as one graft partner (see below). Grafted plants were minimally retarded compared with ungrafted controls, and resumed normal growth rates soon after establishment in pots (Figure 2b). At maturity, plants retained a visible graft union (Figure 2c).

Graft union formation under long days (LD) and short days (SD) was compared in seedlings grafted with collars on nutrient agar. Delayed bolting under SD allowed plants to be maintained in Petri dishes for extended periods. However, early bolting under LD allowed scoring of branching phenotype after 2, rather than 5–6, months. Plants grafted under LD were transferred to a higher temperature (27°C), which was previously reported to promote callus formation and hence development of graft unions (Rhee and Somerville, 1995). Success rate under such conditions was higher than under SD or under lower temperatures, with up to 95% of all grafts taking. In addition, under these conditions the number of plants developing adventitious roots from the scion was only 15–25%, fewer than generally observed for the other methods evaluated. The final proportion of successful grafts in this experiment was therefore over 70%.

Two-shoot grafts

Construction of plants with two different shoots on a single root system was achieved by Y-grafting procedure (Figure 1c). This was a variation on the wedge grafts described above. A shallow-angled lateral cut was made in the hypocotyl of one seedling without severing the root. Into this slit was placed a wedge-cut scion of the second plant. Three-quarters of one cotyledon was removed from each shoot to facilitate alignment of the graft. Grafted plants were then handled and observed exactly as for single grafts. The success rate was similar to single-wedge grafts, although some plants were subsequently discarded where there was a clear imbalance in vigour between the two shoots.

Verification of graft integrity

Visual inspection of grafts allowed early detection and removal of adventitious roots formed on scions. In older plants with well developed, leafy rosettes, inspection was difficult without causing damage. The use of CaMV-35S::GUS or RolC::GUS genes as constitutively expressed markers in one graft partner enabled verification of graft integrity at any stage of development. Normally, plants were harvested and stained immediately after collection of final phenotypic data. Staining for GUS activity precisely locates the graft interface both in shoot–root (Figure 3a,b) and in two-shoot grafts (Figure 3c,d). In these particular specimens no adventitious roots were visible. Plants were excluded from data analysis if adventitious roots were present on the scion, or if the two plants in a two-shoot graft had developed as closely adjacent, but separate plants. The latter occurred occasionally in otherwise successful grafts where adventitious roots on the non-grafted shoot became the dominant root system for that shoot, and the original rootstock was the root system for the scion.

Graft-transmissible regulation of branching

Single grafts were constructed for combinations of WT, max1 and max3 plants. There was no difference between Col and GUS-Col plants in branching phenotype, nor in graft-transmissible influences (data not shown), therefore data were pooled for these genotypes. Unless specifically mentioned, WT refers to combined data for Col and GUS plants, with both being grafted to max1 or max3 mutants in each experiment reported here.

Max1 grafting

As expected, max1 self-grafts had extensive branch development from rosette leaf axils, around threefold more than in WT self-grafts. In both cases, phenotypes of self-grafts were indistinguishable from ungrafted controls (Figure 4a). WT scions did not show increased branching when grafted to max1 rootstocks, but WT rootstocks almost completely inhibited branching of max1 scions (Figure 4d). This indicates that presence of a functional MAX1 gene in either root or shoot is sufficient to inhibit shoot branching under LD. In the case of MAX1 in roots, it is clear that a signal must have been transported to the shoot to enable this inhibition.

Max3 grafting

Grafts between all possible combinations of max3 and WT were constructed and grown under either LD or SD. Two different alleles of max3 were used, max3-1 and max3-9, which show identical patterns of branching (data not shown). Under LD, max3/max3 grafts developed three times as many axillary branches from the rosette as did WT/WT plants (Figure 4e). Under SD, the difference was sixfold (Figure 4f). As with the max1 grafts, WT scions grafted onto max3 rootstocks still developed a WT branching pattern under all conditions. Under LD (Figure 4b,e), max3 scions grafted onto WT rootstocks showed inhibited branching, as did equivalent grafts under SD (Figure 5c,f), although rescue under SD was not as complete as in LD. Therefore, as with MAX1, a MAX3 gene present either in shoot or in rootstock was able to regulate shoot branching.

Graft transmission in a two-shoot system

Two-shoot grafts were generated using combinations of WT and max1. When counting branches, great care was taken to ensure that every rosette branch was correctly allocated to each of the two closely adjoining shoots. Grafted plants consisting entirely of WT tissue developed low numbers of rosette branches on both shoots (Figure 5).
There was a marginal but insignificant trend towards fewer branches on the scion than on the other shoot. Plants with a WT and a max1 shoot on a WT rootstock had WT levels of branching in both shoots. The inhibition of branching in the max1 shoot is similar to the result described above with single grafts of max1 scions on WT rootstocks. In contrast, plants with a WT and a max1 shoot on a max1 rootstock displayed low levels of branching on the WT shoot, but high levels on the max1 shoot (Figure 5).

Discussion

Seedling grafting in Arabidopsis

We present here a range of methods for efficient grafting of Arabidopsis seedlings. Constructing grafted plants at this early stage allows experiments to be conducted for the first time on many aspects of long-distance signalling. This represents a substantial advantage over previous techniques for this species, which were restricted to grafting inflorescence stems late in development when most key processes have already been determined (Rhee and Somerville, 1995; Tsukaya et al., 1993). The use of plants carrying a constitutive GUS reporter gene allows visual confirmation of grafting success. Staining for GUS activity is destructive, and would normally be carried out after final phenotypic analysis of the grafted plants. Non-destructive reporters, such as green fluorescent protein, are an alternative for earlier confirmation.

Figure 4. Branching phenotypes of single grafts between WT and max mutants. (a–c) Overall shoot phenotype; (d–f) rosette branch numbers. (a,d) Grafts without collars between WT and max1, grown under LD. Plants were photographed and scored at 52 days post-grafting. Plants on extreme left and right are ungrafted controls. N = 6–17. (b,e) Grafts with collars between WT and max3, grown under LD. Plants were photographed and scored at 44 days post-grafting. N = 8–12. (c,f) Grafts with collars between WT and max3 grown under SD. Plants were photographed and scored at 159 days post-grafting. N = 6–8. Branching is measured as number of lateral shoots from rosette > 10 mm long. Data are means ± SE. Genotype notation is scion/rootstock.

Figure 5. Branching phenotypes of two-shoot grafts between WT and max1 grown under LD. Genotype notation: self-shoot, scion/rootstock. Plants were photographed at 45 days (a) and scored at 59 days (b). The two-shoot systems were parted to facilitate interpretation of the photograph. Scion is right-hand shoot of each graft. Branching is measured as number of rosette laterals > 10 mm long on each shoot. Data are means ± SE. N = 3–8.
Each version of the methods has its own advantages. The use of collars appears to improve the efficiency of grafting, probably by maintaining very close contact between the cut surfaces. This may be of particular benefit for SD-grown plants, which appear to graft with lower efficiency. A comparison of graft formation between plants with and without collars is required to define the optimum method under SD conditions. The spatial control obtained using collars should also facilitate construction of interstock grafts, as has been demonstrated for rms and dad mutants (Foo et al., 2001; Napoli, 1996). Grafting plants without collars allows greater flexibility in graft design, as demonstrated by the construction of Y-shaped grafts containing both WT and max1 scions. The need for accurate alignment of the cut surfaces, as well as steric considerations, preclude the use of tight-fitting collars in such grafts. Therefore, collarless Y-grafting is the only current method suitable for studying shoot/shoot signalling. Wedge grafts without collars were also successful, although they required high precision in construction of the wedge-cut scion. One advantage of this method is a greater surface area of contact, and hence potentially improved graft connection. In addition, the scion is literally wedged into the rootstock and hence the graft is less likely to separate.

**Grafting analysis of max1 and max3**

The rescue of branching in max1 and max3 scions by wildtype rootstocks is the first demonstration of long-distance signalling in Arabidopsis by a grafting approach. Results of single grafts with both max1 and max3 mutants are consistent with those previously demonstrated for the rms1, rms2 and rms5 mutants of pea (Beveridge et al., 1997b; Morris et al., 2001), and for the dad1 mutant of petunia (Napoli, 1996). The results of the two-shoot grafts with max1 are also similar to those shown for rms1 (Foo et al., 2001), and indicate that (a) two completely different branching phenotypes can be sustained on a single root system; (b) a WT shoot is unable to inhibit branching of a max1 shoot, although it inhibits its own branching; and (c) a max1 shoot is unable to promote branching of an adjacent WT shoot. Overall, MAX1, RMS and DAD1 genes do not appear to mediate shoot-to-shoot regulation of branching, but are involved in root-to-shoot signalling. Although there are strong similarities between Arabidopsis, pea and petunia data, and long-distance inhibition of branching appears to be a widespread phenomenon in plants, it is not yet possible to deduce which genes have orthologous relationships across species. Further physiological characterization, for example reciprocal grafts between different mutants, measurement of hormone levels, transport, metabolism and response, together with gene cloning and expression studies, will lead to clarification of functional similarities and differences across these taxa.

**Grafting in the analysis of other developmental processes**

The grafting techniques described here represent a substantial advance on previous methods for Arabidopsis that used bolted plants ≈30 days old. The ability to graft seedlings from 3 days old facilitates experiments to investigate long-distance signalling at almost any stage of development. Single grafts enable simple tests for shoot-to-root and root-to-shoot signalling, which have been implicated in a number of processes, such as co-ordination of nutrition deficiencies (Raghothama, 1999); regulation of hormonal transport (Bangert, 1994; Beveridge et al., 1997a); and stress responses (Davies and Zhang, 1991; Holbrook et al., 2002). Long-distance signalling within the shoot, such as has been recorded in photoperiodic regulation of floral transition (Weller et al., 1997) or systemic defence responses (Kuc, 2001; Metraux, 2001), can also be assayed using the two-shoot Y-grafting method. We envisage these procedures as complementary to molecular characterization of genes. Grafting of mutants should enable rapid assignment of genes to regulation of transmissible signals involved in these developmental and physiological processes. Finally, there is the potential for detection of other, currently unrecognized, long-distance signals with roles in developmental and physiological processes.

**Experimental procedures**

**Plant materials**

Seeds of Arabidopsis thaliana were obtained from Nottingham Stock Centre (Columbia ecotype, Col-0 and max1; Stirnberg et al., 2002). The max3-1 and max3-9 alleles were isolated from the AMAZE En/Spm population (Wisman et al., 1998) and from an EMS-mutagenized population of Col-0 (Stirmberg et al., 2002). Columbia seed containing a CaMV-35S::GUS transgene were kindly provided by Dr J. Botella (University of Queensland).

**Protocol for grafting without collars**

Seed of Arabidopsis thaliana were surface-sterilized in 70% ethanol for 1 min, then in sodium hypochlorite solution (1% available Cl) for 10 min, followed by extensive washes in sterile distilled water. Seeds were sown under axenic conditions in Petri dishes on a layer of Whatman cellulose nitrate filter (type HA pore 0.45 μm) over a single layer of Whatman No. 1 filter paper. Distilled water was added to saturate the filter, then dishes were sealed with Nascofilm. Plates were placed at 4°C in the dark for 2–3 days. Seedlings were then grown with plates oriented vertically at a constant 23°C with an 18h photoperiod supplied by cool white fluorescent tubes (PAR = 120μmol m⁻² s⁻¹). Three principal hypocotyl-grafting procedures were evaluated: (1) transverse cut and butt alignment; (2) wedge-shaped scion into slit in rootstock; and (3) two-shoot Y-grafts with wedge-cut scion inserted into slit in side of hypocotyl of otherwise intact receiver plant. Position of graft on the hypocotyl (upper, middle or lower) was also tested.

Grafts were all performed under a stereomicroscope, generally using seedlings between 3 and 4 days old. Cuts were most easily made with small blades. A No. 15 scalpel blade was satisfactory, but improved precision could be achieved with a 15° Sharpoint microdissecting knife (Cat no. 1034-12, InterFocus Ltd, Haverhill, UK).

Grafts were visually assessed daily for 5 days, and any plants where the graft had detached due to growth or bending were discarded. Incidence of adventitious rooting was noted, and such roots were carefully excised or crushed with forceps as soon as they emerged. Graft connection was assessed from 3 days after grafting by very gently pulling on scion and rootstock. Survival was enhanced by early transfer to autoclaved potting compost (sieved peat/fine sand, 1:1) or steam-sterilized compost comprising Levington No. 2/fine sand/vermiculite (4:1:1) in 50 ml pots in a covered incubator tray. Some grafts were strong enough to move 4 days after grafting, and all successful grafts were transplanted by 7 days.

Monitoring of adventitious rooting on the scion was continued for 2 weeks after transfer to pots. Plants were either grown to maturity in the growth cabinet, or transferred to a glasshouse kept at 23°C day, 20°C night, with a 16 h photoperiod, supplied by tungsten lights, and supplementary actinic light during winter months.

Protocol for grafting using collars

Grafting with silicon tubing collars was performed under both LD and SD conditions. For grafting under SD, seeds were surface sterilized and sown onto Arabidopsis thaliana salts (ATS) as described by Lincoln et al. (1990). Seeds were incubated at 4°C for 2–4 days, then transferred to a growth cabinet at 23°C with an 8 h photoperiod (94–106 μmol m⁻² sec⁻¹). Plants were grown for 7–9 days under these conditions before grafting. Grafting was performed by cutting the rootstock donor perpendicular to the hypocotyl using a Wilkinson sword ‘Classic’ double-sided blade, then inserting the rootstock into a short length of sterile 0.3 mm diameter tubing (SF medical grade silicone tubing). The scion was inserted in a similar manner and inserted into the other end of the tubing until it touched the rootstock. Plants were then returned to the growth cabinet and grown for a further 4–5 weeks. Plants in which the graft had taken were then transferred to 75 mm diameter plant pots containing Klamann Substrat No. 1 compost (Klasmann-Deilmann GmbH, Geestz, Germany) and grown under identical SD conditions until analysis.

For grafting under LD, plants were prepared as for SD conditions. Seedlings were germinated and grown for 6 days in a growth cabinet at 24°C with a 16 h light/8 h dark photoperiod (120 μmol m⁻² sec⁻¹), before grafting as for SD. Grafted plants were then transferred to growth conditions of 27°C with a 16 h light/8 h dark photoperiod (60–70 μmol m⁻² sec⁻¹). Plants were grown for a further 6–7 days before visual confirmation of graft formation and transfer to 5 cm square plant pots containing Klamann Substrat No. 1 compost, and grown under LD conditions until analysis.

Verification of graft integrity

To confirm scion and rootstock integrity, grafts were performed using homozygous CaMV-35S::GUS or RolC::GUS plants as either scion or rootstock. The other plant part was wild-type Columbia or max mutant. Grafted plants were generated as described above, and were harvested after scoring of final phenotypic data. Plants were trimmed down to a length of hypocotyl and root centred around the graft union, and split longitudinally to facilitate vacuum infiltration. Staining solution contained 1 mM X-Gluc and was based on that of Jefferson et al. (1987). Specimens were stained for 3–24 h at 37°C prior to clearing in 70% (v/v) ethanol. Particular attention was paid to tissues around the graft union, looking for GUS-positive adventitious roots in grafts with GUS scions. Such plants were excluded from analysis.

Test for graft-transmissible branching signals in max mutants

Grafted plants were generated as normal using combinations of max1 or max3 with Col or CaMV35S::GUS-Col or RolC::GUS-Col as wild-type lines. Phenotypes were recorded at the times indicated in the results by scoring the number of axillary branches arising from the rosette in each plant.

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