The Plant Journal (2002) **32**, 255–262

TECHNICAL ADVANCE

Micrografting techniques for testing long-distance signalling in *Arabidopsis*

Colin G. N. Turnbull[1,*,†], Jon P. Booker[2,†] and H. M. Ottoline Leyser[2]
[1]*Department of Agricultural Sciences, Imperial College Wye, University of London, Wye, Kent, TN25 5AH, UK, and*
[2]*The Plant Laboratory, University of York, York, YO10 5YW, UK*

Received 28 March 2002; revised 19 June 2002; accepted 20 June 2002.
*For correspondence (fax +44 20 759 42919, e-mail c.turnbull@ic.ac.uk).
†These two authors contributed equally to the work reported in this paper.

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

© 2002 Blackwell Publishing Ltd

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 *rms5*) 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

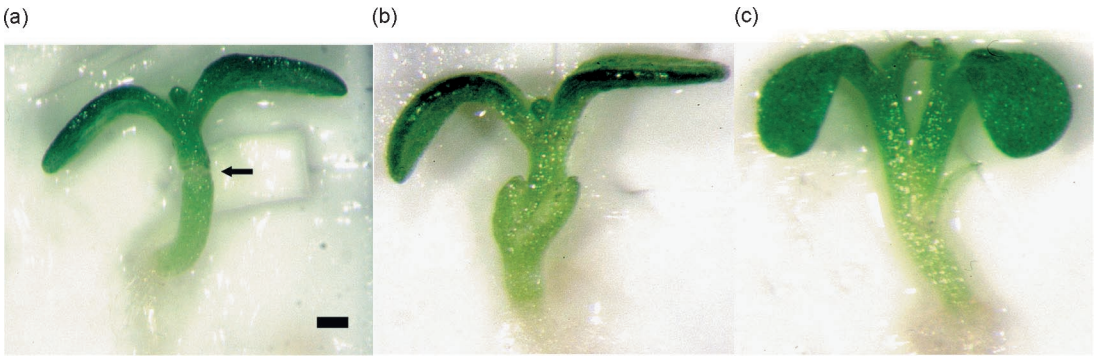

Figure 1. *Arabidopsis* plants 4 days after grafting.
Graft types are 90° butt graft (a); wedge graft (b); two-shoot Y-graft (c). Scale bar, 200 μm. Arrow indicates position of graft union in (a).

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

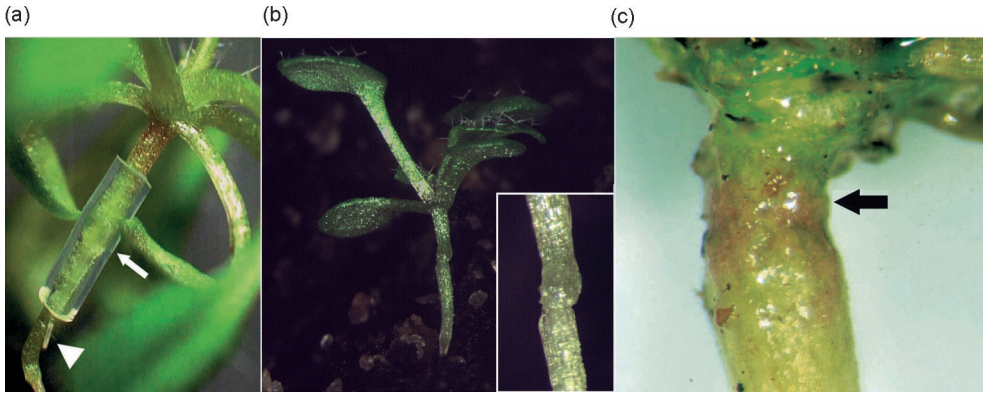

Figure 2. Grafted *Arabidopsis* plants later in development.
Arrows indicate location of graft union.
(a) Butt graft union showing collar on plant 10 days after grafting. Arrowhead indicates adventitious root emerging from base of collar.
(b) Plant growing in pot 14 days after grafting. Inset shows detail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

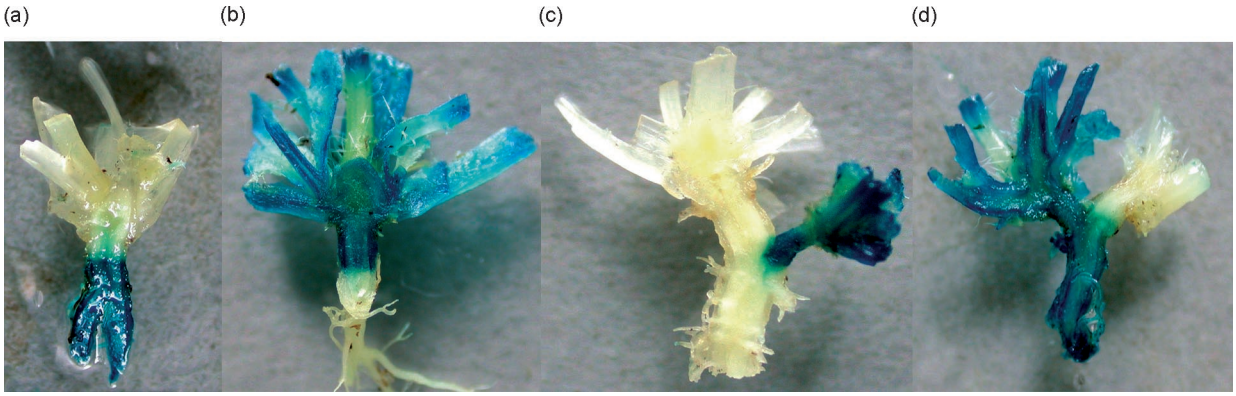

Figure 3. Mature, grafted *Arabidopsis* plants stained with X-Gluc to visualise β-glucuronidase activity.
One partner in each graft carried a CaMV-35S::GUS gene. (a) WT scion on GUS rootstock; (b) GUS scion on WT rootstock; (c) GUS scion in Y-graft onto WT plant; (d) WT scion in Y-graft onto GUS plant. 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 a 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).

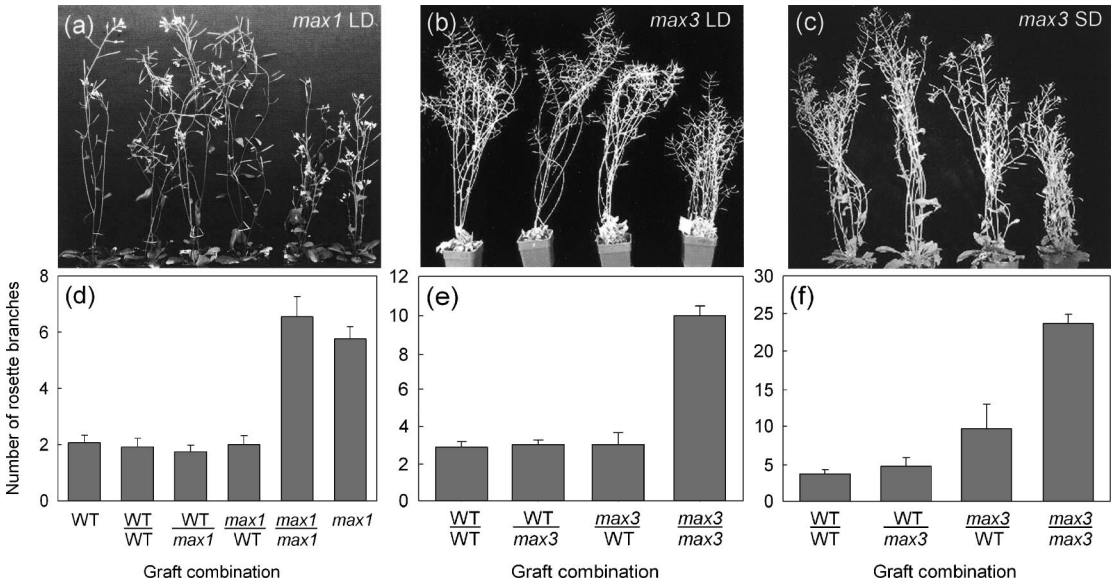

Figure 4. Branching phenotypes of single grafts between WT and *max* mutants.
(a–c) Overall shoot phenotype; (d–f) rosette branch numbers. (a,d) Grafts without collars between WT and *max1*, grown under LD. Plants were photographed and scored at 52 days post-grafting. Plants on extreme left and right 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.

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).

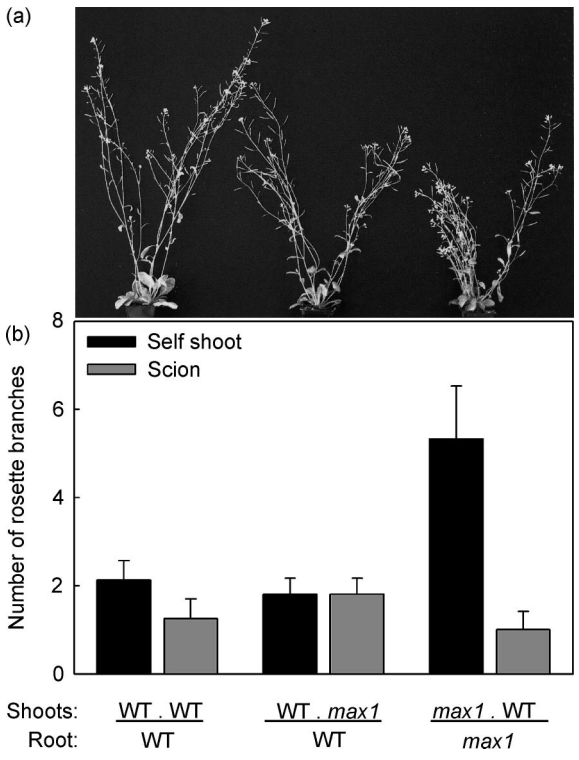

Figure 5. Branching phenotypes of two-shoot grafts between WT and *max1* grown under LD.
Genotype notation: self-shoot . scion / rootstock. Plants were photographed at 45 days (a) and scored at 59 days (b). The two-shoot systems were parted to facilitate interpretation of the photograph. Scion is right-hand 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.

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.

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 wild-type 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 (Bangerth, 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 (Kuć, 2001; Métraux, 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 (Stirnberg *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 Millipore 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 Nescofilm. 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 18 h photoperiod supplied by cool white fluorescent tubes (PAR ≈120 μmol m^{-2} sec^{-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. 10315-12, 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 excised 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 4–6 weeks. Plants in which the graft had taken were then transferred to 75 mm diameter plant pots containing Klasmann 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 Klasmann 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.

Acknowledgements

This work was supported by a Royal Society grant to C.T., and by a BBSRC grant to O.L. We thank Gillian Laird and Norma Wilson for technical assistance, and the University of York horticultural team for plant care.

References

Bangerth, F. (1994) Response of cytokinin concentration in the xylem exudate of bean (*Phaseolus vulgaris* L.) plants to decapitation and auxin treatment, and relationship to apical dominance. *Planta*, **194**, 439–442.

Beveridge, C.A. (2000) Long-distance signalling and a mutational analysis of branching in pea. *Plant Growth Regul.* **32**, 193–203.

Beveridge, C.A. and Murfet, I.C. (1996) The *gigas* mutant in pea is deficient in the floral stimulus. *Physiol. Plantarum*, **96**, 637–645.

Beveridge, C.A., Ross, J.J. and Murfet, I.C. (1994) Branching mutant *rms2* in *Pisum sativum*. Grafting studies and endogenous indole-3-acetic acid levels. *Plant Physiol.* **104**, 953–959.

Beveridge, C.A., Ross, J.J. and Murfet, I.C. (1996) Branching in pea. Action of genes *Rms3* and *Rms4*. *Plant Physiol.* **110**, 859–865.

Beveridge, C.A., Murfet, I.C., Kerhoas, L., Sotta, B., Miginiac, E. and Rameau, C. (1997a) The shoot controls zeatin riboside export from pea roots. Evidence from the branching mutant *Rms4*. *Plant J.* **11**, 339–345.

Beveridge, C.A., Symons, G.M., Murfet, I.C., Ross, J.J. and Rameau, C. (1997b) The *rms1* mutant of pea has elevated indole-3-acetic acid levels and reduced zeatin riboside content but increased branching controlled by graft-transmissible signal(s). *Plant Physiol.* **115**, 1251–1258.

Booker, J.P., van de Sande, K. and Leyser, H.M.O. (1999) *max3*, an *Arabidopsis* mutant with a modified pattern of aerial branching. *Biologia Plant.* **42**, S41.

Bradford, K.J. and Yang, S.F. (1980) Xylem transport of 1-aminocyclopropane-1-carboxylic acid, an ethylene precursor, in waterlogged tomato plants. *Plant Physiol.* **65**, 322–326.

Chailakhyan, M.K. (1968) Internal factors of plant flowering. *Annu. Rev. Plant Physiol.* **19**, 1–36.

Cline, M.G. (1991) Apical dominance. *Bot. Rev.* **57**, 318–358.

Davies, W.J. and Zhang, J. (1991) Root signals and the regulation of growth and development in drying soil. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42**, 55–76.

Foo, E., Turnbull, C.G.N. and Beveridge, C.A. (2001) Long-distance signalling and the control of branching in the *rms1* mutant of pea. *Plant Physiol.* **126**, 203–209.

Haughn, G.W., Schultz, E.A. and Martinez-Zapater, J.M. (1995) The regulation of flowering in *Arabidopsis thaliana*: meristems, morphogenesis and mutants. *Can. J. Bot.* **73**, 959–981.

Holbrook, N.M., Shashidhar, V.R., James, R.A. and Munns, R. (2002) Stomatal control in tomato with ABA-deficient roots: response of grafted plants to soil drying. *J. Exp. Bot.* **53**, 1503–1514.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions – β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901–3907.

Kuć, J. (2001) Concepts and direction of induced systemic resistance in plants and its application. *Eur. J. Plant Pathol.* **107**, 7–12.

Lang, A., Chailakhyan, M.Kh. and Frolova, L.A. (1977) Promotion and inhibition of flower formation in a day-neutral plant in grafts with a short-day plant and a long-day plant. *Proc. Natl. Acad. Sci. USA*, **74**, 2412–2416.

Leyser, H.M.O., Pickett, F.B., Dharmasiri, S. and Estelle, M. (1996) Mutations in the *AXR3* gene of *Arabidopsis* result in altered auxin response including ectopic expression from the *SAUR-AC1* promoter. *Plant J.* **10**, 403–413.

Lincoln, C., Britton, J.H. and Estelle, M. (1990) Growth and development of the *axr1* mutants of *Arabidopsis*. *Plant Cell*, **2**, 1071–1080.

Métraux, J.-P. (2001) Systemic acquired resistance and salicylic acid: current state of knowledge. *Eur. J. Plant Pathol.* **107**, 13–18.

Morris, S.E., Turnbull, C.G.N., Murfet, I.C. and Beveridge, C.A. (2001) Mutational analysis of branching in pea (*Pisum sativum* L.): evidence that *Rms1 and Rms5* regulate the same novel signal. *Plant Physiol.* **126**, 1205–1213.

Murfet, I.C. (1971) Flowering in *Pisum*: reciprocal grafts between known genotypes. *Aust. J. Biol. Sci.* **24**, 1089–1101.

Napoli, C. (1996) The highly branched phenotype of the *Petunia hybrida dad1-1* mutant is reversed by grafting. *Plant Physiol.* **111**, 27–37.

Napoli, C.A., Beveridge, C.A. and Snowden, K.C. (1999) Reevaluating concepts of apical dominance and the control of axillary bud outgrowth. *Curr. Topics Dev. Biol.* **44**, 127–169.

Napoli, C.A. and Ruehle, J. (1996) New mutations affecting meristem growth and potential in *Petunia hybrida*. *J. Heredity*, **87**, 371–377.

Raghothama, K.G. (1999) Phosphate acquisition. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50**, 665–693.

Rhee, S.Y. and Somerville, C.R. (1995) Flat-surface grafting in *Arabidopsis thaliana*. *Plant Mol. Biol. Rep.* **13**, 118–123.

Schurr, U., Gollan, T. and Schulze, E.D. (1992) Stomatal response to drying soil in relation to changes in the xylem sap composition of *Helianthus annuus*. 2. Stomatal sensitivity to abscisic acid imported from the xylem sap. *Plant Cell Environ.* **15**, 561–567.

Stirnberg, P., van de Sande, K. and Leyser, H.M.O. (2002) *MAX1* and *MAX2* control shoot lateral branching in *Arabidopsis*. *Development*, **129**, 1131–1141.

Talbert, P.B., Adler, H.T., Parks, D.W. and Comai, L. (1995) The *REVOLUTA* gene is necessary for apical meristem development and for limiting cell divisions in the leaves and stems of *Arabidopsis thaliana*. *Development*, **121**, 2723–2735.

Thompson, D.S., Wilkinson, S., Bacon, M.A. and Davies, W.J. (1997) Multiple signals and mechanisms that regulate leaf growth and stomatal behaviour during water deficit. *Physiol. Plant*, **100**, 303–313.

Tsukaya, N., Naito, S., Rédei, G. and Komeda, Y. (1993) A new class of mutations in *Arabidopsis thaliana*, *acaulis1*, affecting the development of both inflorescences and leaves. *Development*, **118**, 751–764.

Weller, J.L., Reid, J.B., Taylor, S.A. and Murfet, I.C. (1997) The genetic control of flowering in pea. *Trends Plant Sci.* **2**, 412–418.

Wisman, E., Hartmann, U., Sagasser, M., Baumann, E., Palme, K., Hahlbrock, K., Saedler, H. and Weisshaar, B. (1998) Knock-out mutants from an En-1 mutagenized *Arabidopsis thaliana* population generate phenylpropanoid biosynthesis phenotypes. *Proc. Natl Acad. Sci. USA*, **95**, 12432–12437.