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BREEDING, GENETICS, & GENOMICS

A Grafting Procedure for *Gossypium hirsutum* L. Based on *In Vitro* Grown Seedlings

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ABSTRACT

Intervarietal grafting, and in some cases interspecific grafting, practiced mainly in horticultural crops has many practical applications. Grafting can also be a useful tool to perform basic physiological, biochemical, and molecular studies to better understand communication between shoot and root in a row crop, such as cotton. We have established a grafting protocol for upland cotton (*Gossypium hirsutum* L.) genotypes. Although less efficient, it can be used to create grafts between upland and Pima (*G. barbadense*) or Old-world cottons (*G. arboreum* or *G. herbaceum*). The procedure used 8-day-old seedlings grown in culture under sterile conditions to obtain the scion and rootstock. The scion consisted of the shoot apical meristem with a few leaf primordia, epicotyl, and a small portion of the hypocotyl with the cotyledons removed. The rootstock retained 2 to 3 cm of basal hypocotyl portion with the roots trimmed. Cleft grafts were assembled under aseptic conditions followed by their culture *in vitro*. Once established, as indicated by the healthy growth of three to four true leaves and roots, the grafts were moved to soil in small pots. After hardening the grafts on a laboratory bench for few weeks, they were transferred to larger pots and grown to maturity in a greenhouse to obtain seeds. The success rate using our protocol ranged from 90 to 100% for Coker 312 genotype and its derivative transgenic and gene edited lines. The method was also applicable to generate intervarietal and interspecific graft unions among various types of cotton plants.

Grafting, known to occur naturally, is also practiced widely through human mediation. The grafting process in plants involves cutting and joining of parts of different varieties or species, which following successful establishment of vascular continuity, results in the creation of a chimeric plant. The practice has been in use for thousands of years by various civilizations (Melnyk and Meyerowitz, 2015; Mudge et al., 2009) and currently is used routinely by many horticulturalists. For several species, the process of grafting has largely been automated for industrial-scale production of chimeric plants (Kümpers and Bishopp, 2015). Usually, the procedure uses the shoot of one plant (scion) that is grafted onto the basal part of another plant from which some or most of the shoot portion has been removed (stock or rootstock). Most grafting procedures involve plants that are growing in soil. After the placement of scion onto the stock, the graft union is stabilized using some type of physical pressure, aided by a clip or tape, to promote successful graft formation. Except for the Solanaceae family, grafting between distant family members has little chance of success. Among flowering plants, grafting within a species has been successful only in some basal angiosperms and eudicots. Practical applications of grafting include altering the growth of a tree, avoidance of juvenility, asexual propagation of some woody fruit trees, and conferring resistance to biotic or abiotic stresses. In Asia and some European countries, grafting is practiced for the control of certain fungal diseases in some vegetable/fruit crops (Bletsos and Olympios, 2008; King et al., 2008). Although it is impractical to use grafting techniques for such direct applications in a row crop like cotton, efficient grafting procedures can prove useful to conduct basic scientific investigations. These include examination of long-distance movement of proteins, RNAs, hormones, and other biochemicals. Grafting between two different genotypes or related species also can be used to dissect the role of root and shoot in drought and salinity tolerance (Alves et al., 2021; Coban et al., 2020; Kumar et al., 2017; Liu et al., 2014; Semiz and Suarez, 2019; Silva et al., 2018; Zhang et al., 2019). Similarly, grafting between disease-resistant and susceptible genotypes/species has been used to assess the contribution of root versus shoot in biotic stress tolerance (Couttolenc-Brenis et al., 2021; Huang et al., 2019; King et al., 2008).

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Recent studies on graft formation in *Arabidopsis* have shown that there is temporal separation between the attachment of scion and stock, phloem connection, root formation, and xylem connection (Melnyk and Meyerowitz, 2015; Melnyk et al., 2018). Some genes activated on either side of the graft junction that promote vascular reconnection have been identified (Melnyk and Meyerowitz, 2015). These include auxin- and sugar-responsive genes. Interestingly, the cells in the vicinity of a graft union have been found to exchange genetic materials between the scion and stock (Bock, 2017; Fuentes et al., 2014; Goldschmidt, 2014; Gurdon et al., 2016; Hertle et al., 2021; Li et al., 2013; Stegemann and Bock, 2009). These studies suggest that successful graft union is not simply a formation of stock/scion continuum, but a highly dynamic process. Therefore, it is of value to establish an efficient grafting procedure for a given species of interest to facilitate the study of underlying molecular and biochemical activities resulting in graft union; to investigate the role of root versus shoot in response to various environmental factors and biotic challenges; to study the movement of mineral ions, signaling molecules, and various metabolites between root and shoot; and to examine the exchange of genetic materials between scion and stock cells at the graft union.

Successful grafting between soil-grown cotton plants was demonstrated by Rea (1931), and recently, Ye et al. (2023) used a similar method to examine gossypol biosynthesis and gland formation in cotton plants. Graftings onto rootstocks obtained from soil-grown cotton plants also have been used to rescue transgenic cotton lines (Wang et al., 2019). Successful interspecific grafting between soil-grown *Gossypium hirsutum* L., *G. barbadense* L., and *G. herbaceum* L. lines was shown by Karaca et al. (2020). Interspecific grafting between cotton and sunflower was used by Zhao et al. (2020) to examine the site of gossypol biosynthesis in the cotton plant. Cotton plants grown *in vitro* were used to create successful grafts and to rescue somatic embryos that show poor root formation (Jin et al., 2006; Luo and Gould, 1999). Various research and practical applications of grafting in cotton have been described in a recent review by Zhang et al. (2022).

Our attempts at grafting in cotton plants were initially motivated by the need to investigate whether roots are the main source of gossypol, which is then transported to the above-ground parts of the plant, such as the leaves and seeds, where it is stored within the glands. However, our early attempts to establish grafts between soil-grown, 3-wk-old cotton plants had limited success. This was largely due to the need to maintain high humidity to prevent desiccation of the scion, which eventually resulted in fungal contamination at the graft junction. Therefore, we attempted grafts between 8-d-old seedlings that were grown on germination media in jars under sterile conditions. We describe, in detail, a grafting procedure based on *in vitro* grown cotton seedlings, with success rates ranging from 90 to 100% for grafts between Coker 312 and its derivative transgenic and gene-edited lines. As shown by some other investigators (Banerjee et al., 2000; Chaudhary et al., 2003; Jin et al., 2006; Luo and Gould, 1999), it should be possible to rescue transgenic cotton lines that following somatic embryogenesis were slow or unable to grow into plantlets with healthy roots capable of surviving transfer to soil.

MATERIALS AND METHODS

Plant Material. In all grafting experiments, cotton (*G. hirsutum*) cv. Coker 312 was the common genotype. It is widely used for genetic modification and gene editing. Scions and/or rootstocks consisted of Coker 312 (Coker), TAM66274 [an RNAi-mediated, ultra-low gossypol cottonseed event (ULGCS) (Rathore et al., 2020)]; LCT237 [a CRISPR-mediated knockout event devoid of glands (KO-gl) (Janga et al., 2019)]; and LCT29 [a GFP overexpression event (GFP-cot) (Sunilkumar et al., 2002)].

Grafting and Growth Conditions. Following sterilization of cottonseeds, seed kernels were placed in jars on germination medium (MSO; Murashige and Skoog salts, 2% glucose, pH 5.8, 0.2% phytagel) and grown as described by Rathore et al. (2015). Eight-d-old seedlings were then prepared as stocks or scions under aseptic conditions. For rootstock preparation, the seedling was removed from the jar, excess roots were trimmed, and the hypocotyl was cut 2 to 3 cm above the root/shoot junction. A slit was created at the top end of the truncated hypocotyl by slicing longitudinally to a depth of approximately 5 mm with a sharp scalpel. The scion was prepared from another seedling by first removing the cotyledons and then cutting the hypocotyl approximately 1 cm below the cotyledonary node junction. Removal of the cotyledons from scion is helpful because their weight tends to make the scion top-heavy and the graft union

unsteady. The base of this scion was then trimmed with a scalpel to create an approximately 4-mm long wedge. The wedge part of the scion was then positioned firmly into the slit cut on top of the truncated hypocotyl portion of the rootstock to create a cleft graft. Unlike other reports, no attempt was made to stabilize the graft junction with parafilm or by other means. This entire graft assembly was then placed in a jar containing fresh MSO medium (Rathore et al., 2015). Grafts thus prepared were transferred to a growth room under indirect light for a week before transferring to direct lighting conditions. After 4 to 5 wk following the grafting when several true leaves emerged from the scion, grafted plants were removed from the jars, the germination medium clinging to the roots was washed away under a gentle stream of tap water and were transferred to soil in 1-L pots. Grafted plants were maintained on a laboratory bench under ambient light and temperature with some supplemental incandescent lighting under a humidome for 7 to 10 d and then for another 2 to 3 wk without the humidome. A graft was considered successful when these plants continued to grow following transfer to soil. After hardening the grafts in the laboratory, they were transferred to 19-L (5-gal) pots in a greenhouse and grown to maturity. The step-by-step grafting procedure for cotton is depicted in Fig. 1.

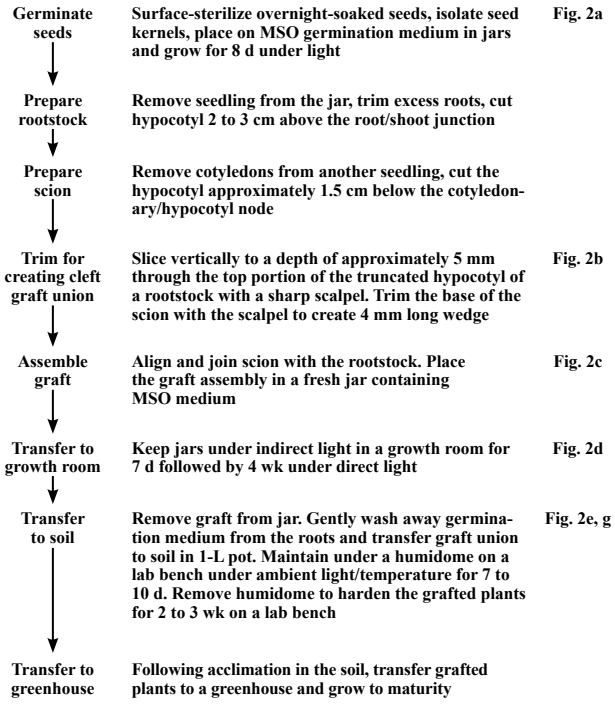

Figure 1. Flow chart outlining the key steps in the grafting procedure using *in vitro* grown cotton seedlings.

Sample Preparation, Sectioning, and Microscopy. A segment of the stem that includes the graft union was cut from a plant 10-wk post-grafting and fixed with a fixative (FAA; formaldehyde:alcohol:acetic acid:water; 10:50:5:35) and embedded in a paraffin block as described by Vitha and Osteryoung (2011). The paraffin-embedded graft unions were sectioned longitudinally (10 µm) with a microtome. The sections were deparaffinized and cleared in 100% xylene. The specimens were stained with Alcin blue 8GX (#ScAn-12) and Safranin O (#AcS-32) and mounted as described by Graham and Trentham (1998). Stained sections were observed under a microscope (Leica DM 6B) and the images were captured using a Leica DM4500 5-megapixel color camera.

RESULTS

For the initial attempts to create grafts, we utilized 3-wk-old plants growing in soil. Even under milder ambient laboratory conditions, the scion leaves showed severe wilting. Although covering the grafted plants with a humidome to maintain high humidity reduced the wilting and allowed most plants to survive, over the following days, the high humidity condition resulted in fungal contamination at the graft junction. Eventually most of these grafts were lost. Therefore, grafting between stocks and scions obtained from cultured seedlings growing under sterile conditions was attempted. Figure 2a shows an 8-d-old seedling removed from the culture jar. Various stages of the preparation of stock and scion and creation of cleft graft are shown in Fig. 2b and c. Assembled grafts were then cultured on MSO medium in jars to allow for the establishment of graft union, indicated by the emergence of leaves and growth of roots (Fig. 2d). Once established in the jars, the grafts were moved to soil in small, 1-L pots. Figure 2e shows a grafted plant following successful establishment on MSO medium and removed from the jar prior to transfer to soil. An image of the established graft union is presented in Fig. 2f showing callus formation at the union site. An example of successful graft following transfer and establishment in soil is shown in Fig. 2g and its union site in Fig. 2h. Sections through successful reciprocal graft unions between wild-type (Coker 312) and GFP-expressing [CaMV35S-P::GFP (GFP-Cot)] plants are shown in Fig. 2i, j.

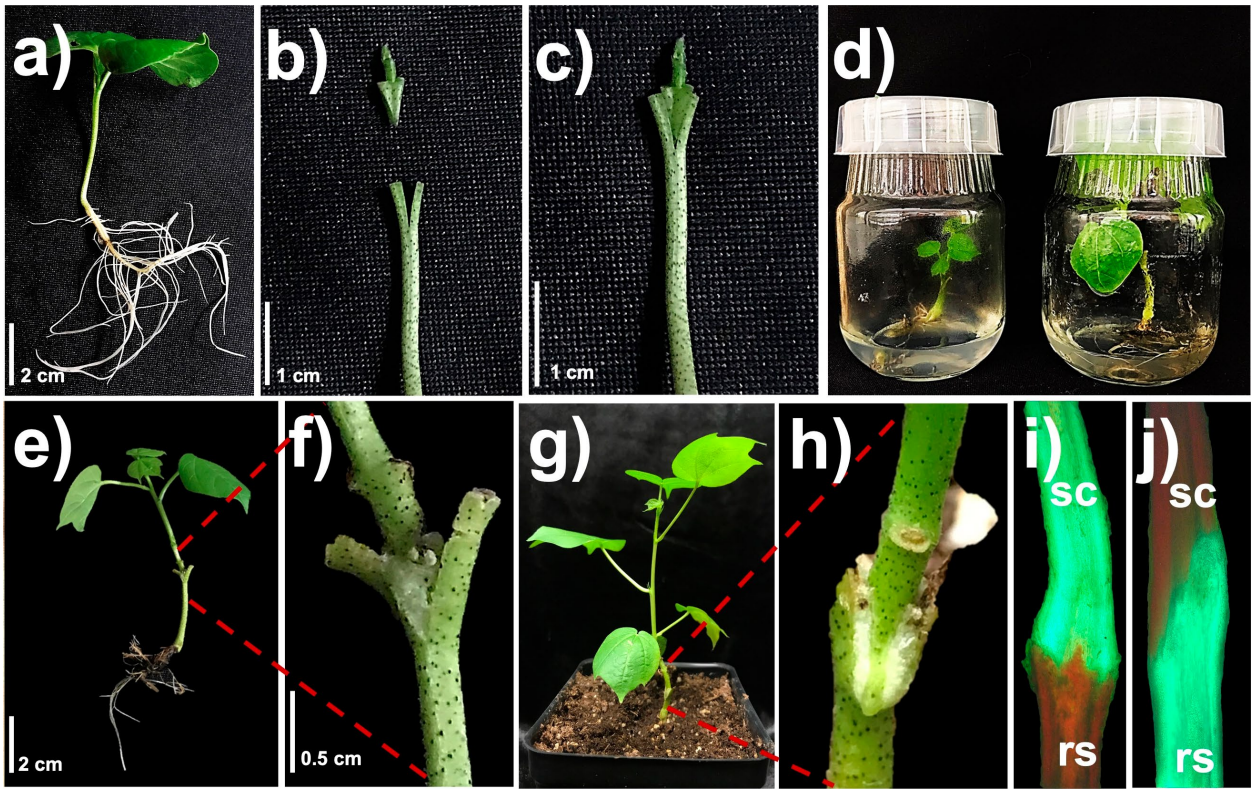

Figure 2. Images showing preparation of rootstocks and scions, grafting and various stages of successful grafted unions (a-h). The red dotted lines indicate the magnified area of the graft junction. Fluorescence images of sections through reciprocal graft unions between wild-type (Coker 312) and CaMV35S-P:GFP plants (i and j). rs: rootstock; sc: scion.

A successful graft requires establishment of a vascular connection between stock and scion. Images of stained longitudinal sections obtained from segments at the graft junction are depicted in Fig. 3. These images clearly show the formation of vascular connectivity between the stock and scion. Our initial objective was to establish a grafting protocol for upland cotton (*G. hirsutum*). Therefore, we utilized cv. Coker 312 (Coker), which is widely used for genetic engineering and gene editing. Most of the grafting experiments were between this genotype and various transgenic and gene-edited lines derived from it. Results from the grafting experiments between various types of *G. hirsutum* seedlings are presented in Table 1. Grafting efficiencies for Coker 312 and its derivative transgenic and gene-edited lines ranged from 90 to 100% using the protocol described in this paper. As an extension of this work, we examined the efficacy of the method to create intervarietal and interspecific grafts. Results for intervarietal grafts [(Coker 312 (Coker), TM1, and Stoneville 474 (STV)] are presented in Table 2. The slightly smaller size of 8-d-old Coker seedlings relative to the other two *G. hirsutum* genotypes was likely responsible for the lower efficiency when this genotype was used as the rootstock. Interspecific grafts were between *G. hirsutum* (Coker) and *G. barbadense* (Gb) and Coker and *G. arboreum* L. (Ga). The *G. barbadense* and *G. arboreum* seeds obtained from USDA-ARS seed repository were old, lacked seed vigor, and the seedlings that did germinate were weaker. Nevertheless, these were used for reciprocal grafting with Coker to evaluate the feasibility of interspecific grafting in cotton. The results presented in Table 3 show successful grafting of these two species with *G. hirsutum* (Coker 312), albeit at lower efficiency. The weakness of *G. barbadense* and *G. arboreum* seedlings is evident based on grafting efficiencies less than half when these two species were used as the rootstock compared to when these were used as scions. Further research is needed to confirm whether differences in seedling vigor played a role in the lower grafting efficiencies when *G. barbadense* and *G. arboreum* were used as root stocks.

The grafts that survived transfer from jars to soil in small pots also survived transfer to larger pots in a greenhouse where they grew normally, produced flowers, and set bolls. Seed cotton was harvested only from four types of grafts: Coker/Coker, ULGCS/Coker, Coker/ULGCS, and ULGCS/KO-gl (Table 4). We used this grafting protocol to establish that the roots are not a source of gossypol for the above-ground parts of a cotton plant, but rather this and related terpenoids are produced and accumulate in an autonomous manner in various shoot organs (Pandeya et al., 2023).

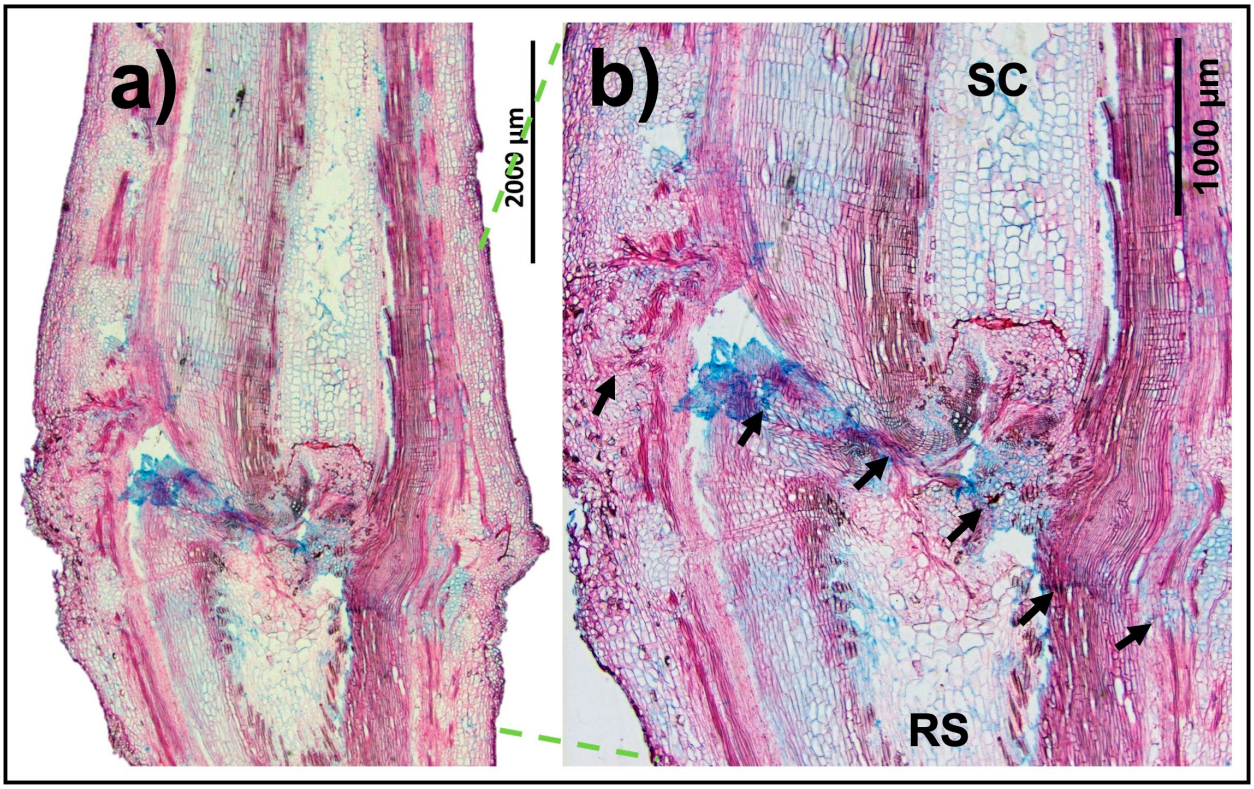

Figure 3. Establishment of a vascular connection in a graft union. A. Longitudinal section of the graft union stained with Alcin blue and Safranin O. B. Magnified portion of the section at the graft union indicated by dotted lines. Arrows indicate the site of union between the rootstock (RS) and scion (SC).

Table 1. Reciprocal grafts between *Gossypium hirsutum*, cv. Coker 312 (Coker) and various transgenic and gene edited events derived from this genotype. ULGCS: an RNAi-mediated, ultra-low gossypol cottonseed event; KO-gl: a CRISPR-mediated knockout event devoid of glands; GFP-cot: a GFP overexpression event

Experiment Number	Type of Graft	# Grafted	# Successful Grafts	% Success
1	Coker/Coker	3	3	89.6%
	ULGCS/Coker	9	9	
	Coker/ULGCS	8	5	
	ULGCS/KO-gl	9	9	
2	Coker/Coker	7	7	100%
	ULGCS/Coker	7	7	
	Coker/ULGCS	7	7	
	ULGCS/KO-gl	7	7	
3	Coker/Coker	10	10	100%
4	Coker/Coker	5	5	95%
	ULGCS/Coker	5	4	
	Coker/ULGCS	5	5	
	ULGCS/KO-gl	5	5	
5	GFP-Cot/Coker	10	8	90%
	Coker/GFP-Cot	10	10	

Table 2. Reciprocal, intervarietal grafts between various *Gossypium hirsutum* genotypes: Coker 312 (Coker), TM1, and Stoneville 474 (STV)

Experiment Number	Type of Graft	# Grafted	# Successful Grafts	% Success
6	TM1/Coker	12	9	75%
	Coker/TM1	12	11	91.7%
7	STV/Coker	12	4	33.3%
	Coker/STV	12	11	91.7%

Table 3. Reciprocal, interspecific grafts between *Gossypium hirsutum*, cv. Coker 312 (Coker) and *G. arboreum* (Ga), and between Coker and *G. barbadense* (Gb)

Experiment Number	Type of Graft	# Grafted	# Successful Grafts	% Success
8	Ga/Coker	20	15	75%
	Coker/Ga	19	6	31.6%
9	Gb/Coker	21	19	90.5%
	Coker/Gb	20	8	40%

Table 4. Seed and fiber yields of various grafts grown under greenhouse conditions. Data represent Mean±SE.

Type of Graft[z]	Seed Yield	Fiber Yield
	g/plant	
Coker/Coker	85.01±4.2 (n=7)	53.48±2.9 (n=7)
ULGCS/Coker	77.42±2.8 (n=11)	42.20±1.3 (n=11)
Coker/ULGCS	83.23±3.4 (n=8)	47.18±3.0 (n=8)
ULGCS/KO-gl	83.21±4.5 (n=8)	46.15±2.5 (n=8)

[z] **Coker: Coker 312; ULGCS: an RNAi-mediated, ultra-low gossypol cottonseed event; KO-gl: a CRISPR-mediated knockout event devoid of glands**

DISCUSSION

Cotton is the largest and most popular natural fiber crop grown by approximately 20 million farmers in many parts of the world. Given its importance as a source of fiber and feed, an efficient grafting system for this crop will be useful in conducting various types of studies to understand the nature of communication between the root and shoot.

The method described herein is efficient in creating grafts between various *G. hirsutum* genotypes. We expect the protocol to be just as effective between various cotton species provided that healthy seedlings with good vigor are used for grafting. The inherent difference in the hypocotyl thickness, for example between various *G. hirsutum* genotypes and between *G. hirsutum* and *G. arboreum* species, can be overcome by using slightly older seedlings of the species that has thinner hypocotyls at the 8-d stage used in the current investigation. Unlike a previous investigation that found complete removal of the roots from the stock to be beneficial (Jin et al., 2006), our study did not encounter any problems with the use of rooted stocks. Trimming of the roots to prepare the rootstock was done only to minimize the chance of contamination while manipulating the cultured seedling in the laminar flow hood. Unlike previous studies (Karaca et al., 2020; Luo and Gould, 1999), the presence of cotyledons, either on scion or stock, was found not to be necessary for a successful graft union. Cotyledons if left intact on a scion can easily cause it to topple because of their heavy weight. Also, the presence of cotyledons on a scion makes it difficult to replant the graft in a standard culture jar and requires a Magenta box or wider glass jar for successful transfer. Another disadvantage of retaining the cotyledons on the rootstock is the possibility of shoots arising from the cotyledonary node that need to be removed post-graft formation (Luo and Gould, 1999). Scions trimmed in the manner described in this paper also did not require stabilization of the graft junction with parafilm (Mushke et al.,

2012; Jin et al., 2006), thus further avoiding chance of contamination during the establishment of the grafts *in vitro*. Banerjee et al. (2000) reported lower survival of grafts when cultured on semi-solid media (65-70%) compared to their culture on liquid media (90-95%). In comparison, our success rate was 90 to 100% for grafts between Coker 312 and derivative genotypes when cultured on MSO medium solidified with 0.2% phytagel.

The method described herein could be useful in prescreening desirable traits inherent to the shoot or root before attempts are made to combine these through breeding that require many years and considerable effort. For example, combining the desirable fiber quality and yield traits of one genotype with drought tolerance of another genotype can be evaluated using grafts grown under field conditions before resources and efforts are invested in breeding. Similarly, early evaluation of superior fiber quality and yield traits of one genotype with nematode resistance of another genotype would save a great deal of time, effort, and resources. One person working for eight hours can easily create 50 to 60 grafts. Thus, a small-scale field trial using approximately 500 grafts is quite feasible.

For a successful graft to occur, the cells of scion and stock first make connections through *de novo*-formed plasmodesmata before making functional vascular connections (Jeffree and Yeoman, 1983). Recent studies on grafting show transfer of plastids from the cells of one plant to another (Hertle et al., 2021; Stegemann and Bock, 2009). Some of the cells of the scion within the freshly formed graft junction contain plastids of the stock and vice versa. Similarly, experimental evidence presented by Gurdon et al. (2016) demonstrated cell-to-cell movement of mitochondria at the graft junction. Interestingly, transfer of whole nuclear genome among the cells at the graft junction leading to creation of a novel species has also been demonstrated (Fuentes et al., 2014). In theory, culture of cells from the graft junction followed by regeneration provides an exciting opportunity to create novel genetic combinations as a result of horizontal organelle and/or nuclear genome transfer. The simple yet efficient grafting procedure for cotton described in this report should facilitate application of such approaches in the future to improve this important crop.

In conclusion, the grafting protocol for cotton described herein can be used to study movement of various biochemicals between root and shoot and to better understand the role and contribution of root and shoot in the tolerance to biotic and abiotic stresses. In addition, a high efficiency grafting protocol between cotton species could become a valuable tool in creating novel genetic combinations resulting from the exchange of nuclear or organelle genetic materials between cells at the graft union. As reported by others (Banerjee et al., 2000; Chaudhary et al., 2003; Jin et al., 2006; Luo and Gould, 1999), use of *in vitro*-grown seedlings for grafting should also find applications in rescuing transgenic lines that are obtained after months through tissue culture and fail to produce viable roots to support the shoot growth and develop into a functioning plantlet. The protocol described in this report can easily be adapted for other important dicot crops such as soybean and sunflower.

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