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Grafting Imparts Glyphosate Resistance in Soybean

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Grafting is a widely-adopted cultural method to incorporate desired traits of rootstock with those of the scion and has been used successfully to address many biotic and abiotic stresses, including drought/waterlogging, insects, and diseases. However, it is not known if a herbicide resistance trait can be transferred across a graft union. Using Roundup Ready® (RR; glyphosate-resistant) soybean grafted with conventional (CN; nontransgenic and glyphosate-sensitive) soybean, we show that grafting is capable of transferring glyphosate resistance from RR rootstocks to CN scions. Grafts of CN/CN (scion/rootstock), CN/RR, RR/CN, and RR/RR were treated with potassium salt of glyphosate at 0.28, 0.84 and 1.68 kg ae ha^{-1}. The CN/RR plants survived glyphosate treatment at 0.84 and 1.68 kg ha^{-1} while CN/CN plants were killed, indicating that glyphosate resistance is systemically mobile across the graft union. Intraspecies transfer of glyphosate resistance was unidirectional from root to shoot, since RR/CN plants were killed by glyphosate. The glyphosate resistance trait is conferred by CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4-EPSPS); therefore, we further examined whether CP4-EPSPS played a role in the phenomenon. CP4-EPSPS was detected in the CN scion of CN/RR plants by enzyme-linked immunosorbent assay (ELISA) but only 0.001% of that detected in RR leaf. This concentration is unlikely to have contributed significantly to the glyphosate resistance observed in CN/RR plants. Amino acid systemic trafficking and/or tissue specific glyphosate resistance are more likely the reasons for this phenomenon. These results show that grafting a transgenic herbicide-resistant rootstock to a nonresistant scion can confer resistance to the entire plant.
Nomenclature: Glyphosate; soybean, *Glycine max* (L.) Merr.
Key words: CP4-EPSPS, gene flow, glyphosate resistance, intraspecies trafficking.

El injertar es una práctica cultural ampliamente adoptada para combinar caracteres deseados de un patrón con aquellos del injerto y ha sido utilizada exitosamente para lidiar con muchos estreses bióticos y abióticos, incluyendo sequía/inundación, insectos y enfermedades. Sin embargo, no se sabe si el carácter de resistencia a herbicidas puede ser transferido a través de la unión del injerto. Usando soya resistente a glyphosate (RR) injertada con soya no resistente a glyphosate (CN), nosotros demostramos que los injertos son capaces de transferir la resistencia a glyphosate de un patrón resistente a tejido convencional injertado. Injertos de CN/CN (injerto/patrón), CN/RR, RR/CN, y RR/RR fueron tratados con sal potásica de glyphosate a 0.28, 0.84 y 1.68 kg ae ha^{-1}. Las plantas CN/RR sobrevivieron al tratamiento con glyphosate a 0.84 y 1.68 kg ha^{-1}, mientras que las plantas CN/CN murieron, lo que indica que la resistencia a glyphosate es móvil sistémicamente a través de la unión en el injerto. En vista de que glyphosate mató a las plantas RR/CN, la transferencia intra-específica de resistencia a glyphosate fue unidireccional desde la raíz al tejido aéreo,. El carácter de resistencia a glyphosate es conferido por CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4-EPSPS), por lo que examinamos si CP4-EPSPS jugó algún rol en el fenómeno observado. por medio de un ensayo de inmunoabsorción ligado a enzimas (ELISA), se detectó CP4-EPSPS en el injerto CN de plantas CN/RR, pero solamente un 0.001% de los niveles detectados en hojas RR. Esta concentración es poco probable que haya contribuido en forma significativa a la resistencia a glyphosate observada en plantas CN/RR. Tráfico sistémico de amino ácidos y/o resistencia a glyphosate en tejidos específicos son probablemente las razones que explican este fenómeno. Estos resultados muestras que injertar tejido sin resistencia a herbicidas sobre un patrón resistente puede conferir resistencia a toda la planta.

Grafting is a common technique in horticulture that is used to combine desired traits of scion and rootstock. In the late 19th century, the European wine industry was rescued from the devastating effects of the soil-borne insect phylloxera by grafting sensitive grapes onto a phylloxera-tolerant rootstock. Grafting continues to be the most important and efficient way to address this pest (Pouget 1990). Grafting is also used to manipulate scion morphology and to manage other biotic stresses including viral, bacterial, and fungal diseases and nematodes, as well as abiotic stresses such as drought/waterlogging and soil alkalinity/acidity (Mudge et al. 2009).

Lusser et al. (2011) recommended development of new transgenic rootstock lines to resist biotic and abiotic stresses for which resistance genes are rare in the plant genome. An advantage of grafting is prevention of gene flow, provided suckers from transgenic rootstocks are removed (Lev-Yadun and Sederoff 2001). Applications to date of transgenic rootstocks include management of fanleaf virus in grapes (Gambino et al. 2005) and fungal diseases in citrus (Mitani et al. 2006). More recently, Haroldsen et al. (2012) reported that the transgenic disease resistant tomato rootstock could reduce pathogen damage in the grafted nontransgenic scion. The potential of grafting to address the abiotic stress of herbicide exposure has not been assessed.

Herbicides and herbicide-resistant plant materials are used on a very large scale. The global adoption of transgenic crops has experienced continuous growth for 16 years, reaching 160 million planted ha in 2011 (James 2012). Approximately

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85% of transgenic crops carry a herbicide-resistant trait, either alone or stacked with other traits. Primary crops include corn (*Zea mays* L.), soybean, cotton (*Gossypium hirsutum* L.), canola (*Brassica napus* L.), and sugar beet (*Beta vulgaris* L.). The glyphosate-resistant trait dominates all other herbicide-resistant traits. Glyphosate kills plants by blocking 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), a key enzyme for synthesis of aromatic amino acids in plants. The RR trait is conferred by the EPSPS gene from *Agrobacterium* spp. strain CP4 (CP4-EPSPS; Barry et al. 1997). Unlike endogenous plant EPSPS, CP4-EPSPS is resistant to glyphosate and thus ensures the synthesis of aromatic amino acids in the RR plants.

We hypothesized that a RR rootstock could confer glyphosate resistance upon grafted conventional (CN; non-transgenic and glyphosate-sensitive) shoots at a level that could be readily detected by glyphosate application. We tested this hypothesis using grafted plants of CN and RR soybean. The specific objective was to determine the response of different grafting progenies to glyphosate application.

Materials and Methods

Grafting Experiment. Soybean was selected as the model system for this study because of the availability of glyphosate-resistant soybean seeds and high survival rate of grafted plants. CN and RR soybean (SC354 and SC9328RR, Seed Consultants Inc., 648 Miami Trace Road SW, Washington Court House, OH 43160) were sown in a seedling flat with 64 cells and propagated in a greenhouse with day/night period of 16/8 h and a corresponding thermoperiod of 30/20 °C. At the unifoliate growth stage, the following graft combinations were created: CN/CN (scion/rootstock), CN/RR, RR/CN and RR/RR. The unifoliate leaf was removed from both rootstock and scion materials. A "V" shaped notch was cut in the rootstalk stem above the cotyledons, and grafted with a wedge-shaped scion bearing the developing leaf shoot. To provide support, polyethylene tubing of 2.5 mm diameter and 1 cm length was cut open on one side and slipped over the graft union. Grafted plants were cultured in the laboratory for 1 wk at room temperature in a plastic-covered tent before moving back to the greenhouse. Two wk later, grafted seedlings were transplanted into 10 cm square pots filled with a 1 : 1 mixture of Wooster silt loam soil and Pro-Mix potting soil (Premier Tech., 1 Avenue Premier, Rivière-du-Loup, QC G5R 6C1, Canada). Plants were grown for an additional 7 d, and herbicide treatments were applied when grafted plants were at 2- to 3-leaf stage.

Protein Detection by ELISA. At the 3-leaf stage, the fully-expanded third leaf was collected from CN/CN and CN/RR plants for CP4-EPSPS extraction. Leaf samples were homogenized in 1.5-ml tubes using the extraction buffer provided by the ELISA kit (Envirologix, 500 Riverside Industrial Parkway, Portland, ME 04103) at 1 : 1 ratio (weight : volume) and centrifuged for 5 min at 13,000 rpm. The supernatant was then transferred to a 1.5-ml tube for ELISA testing. ELISA was conducted following the protocol provided by the kit (Grothaus et al. 2006). Standard curves were prepared based on a series of dilutions (10^3, 10^4, 10^5, and 10^6) of protein extract from the RR leaf. A linear standard curve was achieved at 10^4, 10^5, and 10^6 times dilution of RR leaf extract with a R^2 value of 0.9995. The optical density (OD) values of all samples extracted fell into this linear range.

Herbicide Treatment. Glyphosate was applied with a track-sprayer equipped with a 3-nozzle boom. Grafts of CN/CN, CN/RR, RR/CN and RR/RR were sprayed with potassium salt of glyphosate (Roundup WeatherMax®, Monsanto Company, 800 North Lindbergh Boulevard, St. Louis, MO 63167) at 0.28, 0.84, and 1. 68 kg ae ha^{-1}, equivalent to 1/3, 1, and 2 times the 0.84 kg ha^{-1} rate recommended for weed control in soybean, respectively (Monsanto Company 2009). Nonionic surfactant (Spreader 90, Loveland Products, Inc., 3005 Rocky Mountain Avenue, Loveland, CO 80538) was added to all herbicide solutions at 0.25% v/v. The application pressure was 276 kPa and the volume was 234 L ha^{-1}. The response of grafted plants to herbicide treatments was recorded by measuring plant height, and by photography. Plant height was defined as the length from the cotyledon node to the extended tip of the leaves. Height of plants that were severely injured by glyphosate was not measured because they were too fragile to withstand the manipulation required.

Statistical Analysis. A complete randomized design with 5 replications per treatment was used and the experiment was repeated twice. ELISA and plant height data were subject to an ANOVA model and means were separated by the *t* test at the 0.05 level of significance (SAS 9.3, SAS Institute Inc., 100 SAS Campus Drive, Cary, NC 27513).

Results and Discussion

CN/CN plants treated with glyphosate at 0.84 and 1.68 kg ha^{-1} were dead 7 d after treatment (DAT; Figure 1). Glyphosate at 0.28 kg ha^{-1} injured CN/CN plants, i.e. chlorosis of leaves at 7 DAT. Plants treated with glyphosate at 0.28 kg ha^{-1} did not elongate during the first 14 d (Figure 2b). Thereafter, these plants recovered from the growth suppression effect and by 33 DAT their height was similar to that of RR/RR plants. However, an abnormal leaf shape (narrower leaves) caused by glyphosate was still evident 24 DAT (Figure 1).

CN/RR plants survived all glyphosate treatments regardless of application rate (Figure 1). At 0.28 kg ha^{-1} glyphosate caused chlorosis of the newly expanding trifoliate leaf but older fully expanded leaves were unaffected. At 0.84 and 1.68 kg ha^{-1}, injury became more severe on young leaves and spread to mature leaves; however, at 14 DAT plants treated with these rates of glyphosate were developing new terminal shoots and by 24 DAT these plants had largely recovered from the initial reduction in growth. Nevertheless, these plants still expressed the modified morphology associated with glyphosate injury, specifically a more wrinkled adaxial surface when compared with leaves of RR/RR plants (Figure 3).

Growth of CN/RR plants was affected by glyphosate in a rate-dependent pattern (Figure 2). CN/RR plants did not vertically grow for 14 d when treated with glyphosate at 0.28 kg ha^{-1}, and their height was only 51% of that untreated CN/

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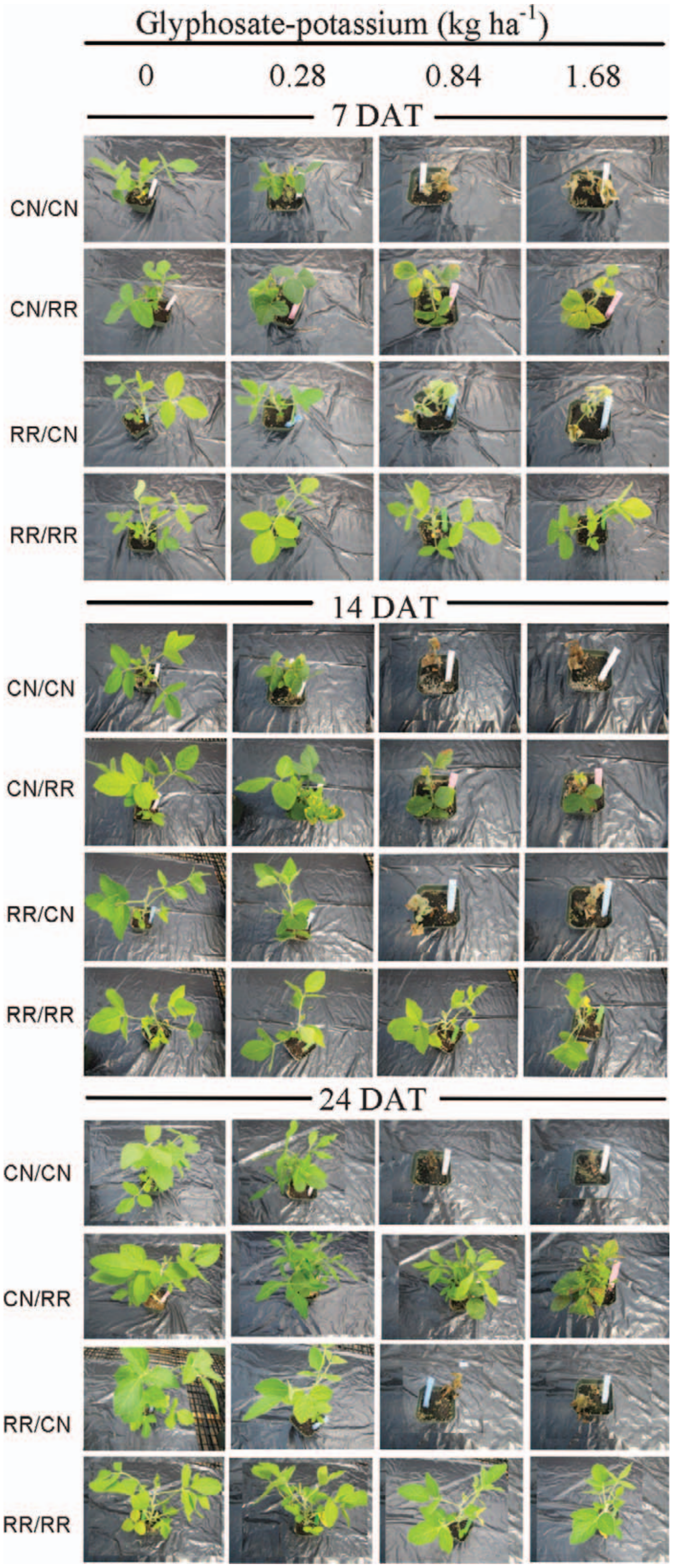

Figure 1. Effect of foliar-applied glyphosate on grafted soybeans at 7, 14 and 24 d after treatment (DAT). Grafted plants are described by their scion/rootstock constructs; for example, CN/RR refers to a soybean plant with a conventional (CN; nontransgenic and glyphosate-sensitive) shoot grafted to a Roundup Ready® (RR; glyphosate-resistant) rootstock. All plants were grown in 10 cm wide square pots.

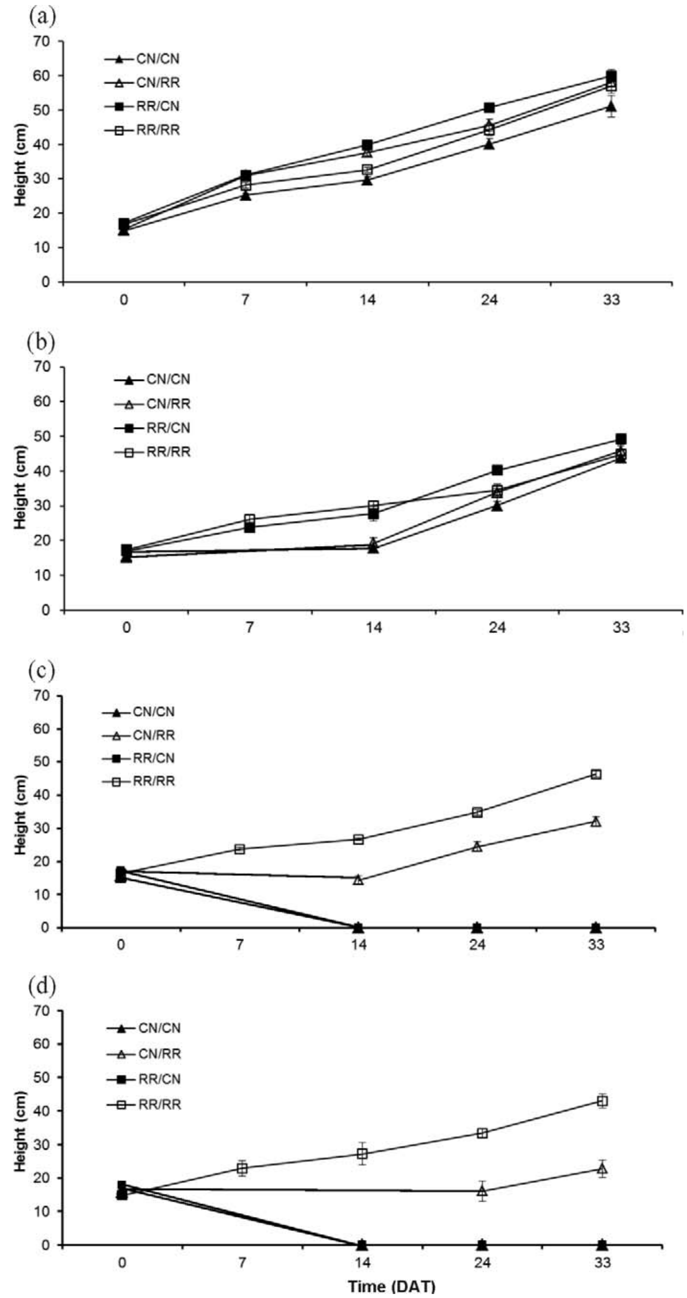

Figure 2. Effect of foliar-applied glyphosate on grafted soybean plant height at 0, 7, 14, 24 and 33 d after treatment (DAT) with 0 (a), 0.28 (b), 0.84 (c) and 1.68 kg ae ha^{-1} (d). Grafted plants are described by their scion/rootstock constructs; for example, CN/RR refers to a soybean plant with a conventional (CN; nontransgenic and glyphosate-sensitive) shoot grafted to a Roundup Ready® (RR; glyphosate-resistant) rootstock. All plants were grown in 10-cm-wide square pots. Height of CN/RR and CN/CN plants treated with glyphosate was not measured at 7 DAT because herbicide injured leaves were too delicate to manipulate. Similarly, the height of CN/RR plants treated with glyphosate at 1.68 kg ha^{-1} was not measured 14 DAT. Height of dead plants was recorded as 0. Vertical bars represent the standard error of the mean (n = 5).

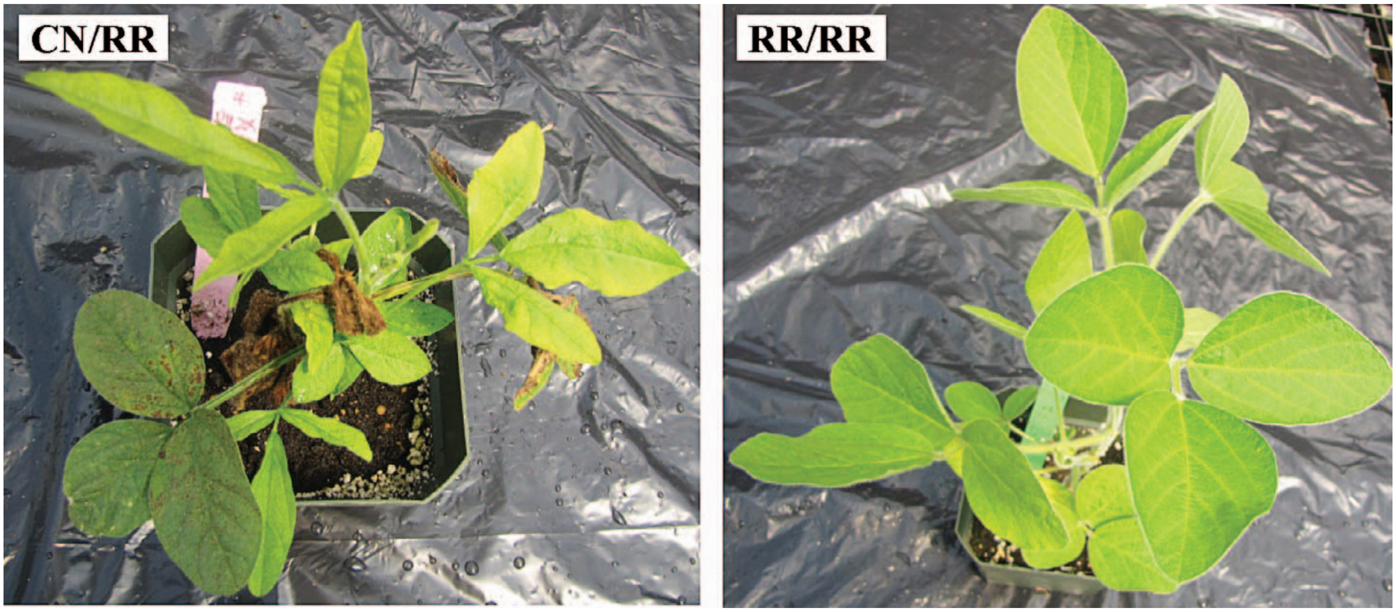

Figure 3. Effect of glyphosate at 1.68 kg ae ha^{-1} on grafted soybean plants at 24 d after treatment. Notice that the new developed leaves of CN/RR plant are narrower and elongated when compared with leaves of RR/RR plants. CN/RR stands for a soybean plant with a conventional (CN; nontransgenic and glyphosate-sensitive) shoot grafted onto a Roundup Ready® (RR; glyphosate-resistant) rootstock, and RR/RR stands for a RR shoot grafted onto a RR rootstock.

RR plants (Figure 2a and 2b). However, CN/RR plants were similar in height to RR/RR plants by 33 DAT, indicating that they had largely recovered from stunting effect of glyphosate. At 0.84 kg ha^{-1}, the vertical growth of CN/RR plants ceased for the first 14 d, and only attained a height that was 75% of RR/RR plants by 33 DAT (Figure 2c). Glyphosate at 1.68 kg ha^{-1} was more injurious as vertical growth was arrested through 24 DAT and plant height was only about 50% of RR/RR plants at 33 DAT (Figure 2d).

Similar to CN/CN plants, RR/CN plants were also killed by glyphosate at 0.84 and 1.68 kg ha^{-1} (Figure 1). However, the mode of lethality appeared to be different from that of CN/CN plants. Glyphosate injury was more advanced on CN/CN plants than on RR/CN; moreover, death of glyphosate-treated RR/CN plants occurred quickly after an apparent initial injury-free period of 5 or 6 d. Plant death resembled dehydration (Figure 4). These results suggest that glyphosate moved from treated shoots to the root, either

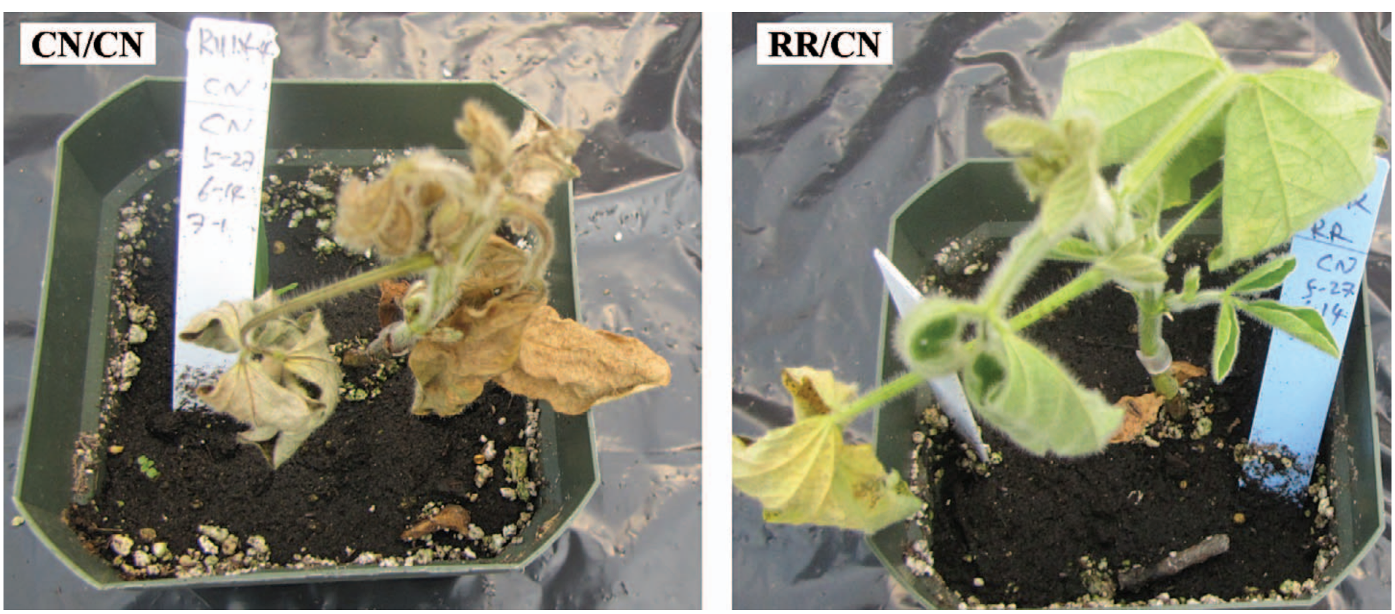

Figure 4. Effect of glyphosate at 0.84 kg ae ha^{-1} on grafted soybean plants at 7 d after treatment. The leaves of CN/CN plants were "burned" by glyphosate; whereas RR/CN plants died in a way similar to dehydration after an initial injury free period of 6 d. CN/CN stands for a soybean plant with a conventional (CN; nontransgenic and glyphosate-sensitive) shoot grafted onto a CN rootstock, and RR/CN stands for a Roundup Ready® (RR; glyphosate-resistant) shoot grafted onto a CN rootstock.

Table 1. CP4-EPSPS quantification by ELISA[a] in leaves of grafted soybeans.

Grafts[b]	CP4-EPSPS relative concentration[c]
	10^{-6} of RR leaf extract
CN/CN	0 (0.1) b
CN/RR	14.8 (3.4) a

[a] Values in parentheses represent the standard error of the mean (n = 6).

[b] Grafting progenies were expressed as scion/rootstock; for example, CN/RR stands for a soybean plant with a conventional (CN; nontransgenic and glyphosate-sensitive) shoot grafted to a Roundup Ready® (RR; glyphosate-resistant)rootstock.

[c] Means followed by the same letter are not significantly different from each other based on *t* test at the 0.05 level.

killing the root system or preventing it from supplying sufficient water for transpiration. Although glyphosate at 0.28 kg ha^{-1} did not cause apparent foliar injury to RR/CN plants, their height was statically the same with that of RR/RR plants at 7 and 14 DAT (Figure 2b). Considering that untreated RR/CN plants were taller than RR/RR plants ($P < 0.05$) during the same period (Figure 2a), this result suggests that glyphosate at 0.28 kg ha^{-1} temporarily reduced the growth of RR/CN plants.

As transgenic CP4-EPSPS exclusively confers glyphosate resistance in RR soybean, we further investigated whether CP4-EPSPS can move from RR roots to CN scions. CP4-EPSPS was detected by ELISA in the leaves of CN/RR plants at approximately 0.001% of that encountered in RR leaves (Table 1). Evaluating the biological significance of low levels of the enzyme was beyond the scope of this research; however, it seems unlikely that such a low level would have contributed significantly to the observed glyphosate resistance. Because amino acids are mobile in both phloem and xylem (Fischer et al. 1998), we suspect that aromatic amino acid trafficking from RR rootstocks to CN scions is a more likely cause of observed glyphosate resistance in CN/RR plants. Aromatic amino acids produced in the RR scion of RR/CN plants would translocate to the CN rootstock via phloem in a source to sink fashion. Given that xylem volume flow is at least 10 times more than phloem volume flow (Windt et al. 2006) and amino acids can move from phloem to xylem (Fischer et al. 1998), amino acids translocated to the root from RR scions would predominantly be swept back to shoot tissue by the transpiration stream. Overtime the net deficit in amino acids in the root system would lead to death of those tissues and the concomitant death of the scion by water deprivation. In addition, greater soybean shoot tissue resistance of glyphosate than root tissue is perhaps because of a higher expression of endogenous plant EPSPS and/or a higher efficiency of metabolizing glyphosate in the shoot.

In conclusion, this research demonstrates a novel expression of transgenic herbicide resistance that does not require inclusion of the transgene in the reproductive portion of the plant. Although soybean is not an economically feasible crop for grafting, many other annual vegetables such as tomato and cucurbits have incorporated grafting as an effective tool to manage different bio-stresses. Our results indicated that glyphosate resistance is a mobile trait that could be a target of grafting practice for these annual vegetables. However, further research is required to address limitations of the current experiments including whether glyphosate resistance in grafted CN scions will be "diluted" as plants grow bigger, and whether the herbicide application affects crop yield. The utility of other herbicide resistant traits in grafted plants should also be addressed. As grafting can completely prevent gene flow (Haroldsen et al. 2012; Lev-Yadun and Sederoff 2001), it holds promise as a means to utilize transgenic phenotypes while preventing hybridization between transgenic crops and their closely related wild species.

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