Glyphosate-Induced Anther Indehiscence in Cotton Is Partially Temperature Dependent and Involves Cytoskeleton and Secondary Wall Modifications and Auxin Accumulation

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Yield reduction caused by late application of glyphosate to glyphosate-resistant cotton (Gossypium hirsutum; GRC) expressing CP4 5-enol-pyruvylshikimate-3-P synthase under the cauliflower mosaic virus-35S promoter has been attributed to male sterility. This study was aimed to elucidate the factors and mechanisms involved in this phenomenon. Western and tissue-print blots demonstrated a reduced expression of the transgene in anthers of GRC compared to ovules of the same plants. Glyphosate application to GRC grown at a high temperature regime after the initiation of flower buds caused a complete loss of pollen viability and inhibition of anther dehiscence, while at a moderate temperature regime only 50% of the pollen grains were disrupted and anther dehiscence was normal. Glyphosate-damaged anthers exhibited a change in the deposition of the secondary cell wall thickenings (SWT) in the endothecium cells, from the normal longitudinal orientation to a transverse orientation, and hindered septum disintegration. These changes occurred only at the high temperature regime. The reorientation of SWT in GRC was accompanied by a similar change in microtubule orientation. A similar reorientation of microtubules was also observed in Arabidopsis (Arabidopsis thaliana) seedlings expressing green fluorescent protein tubulin (tubulin a 6) following glyphosate treatment. Glyphosate treatment induced the accumulation of high levels of indole-3-acetic acid in GRC anthers. Cotton plants treated with 2,4-dichlorophenoxyacetic acid had male sterile flowers, with SWT abnormalities in the endothecium layer similar to those observed in glyphosate-treated plants. Our data demonstrate that glyphosate inhibits anther dehiscence by inducing changes in the microtubule and cell wall organization in the endothecium cells, which are mediated by auxin.

Glyphosate resistance has consistently been the dominant trait in commercial transgenic crops, encompassing 72% of the genetically modified crops grown in 17 countries worldwide (James, 2005). Glyphosate is a competitive inhibitor of the 5-enol-pyruvylshikimate-3-P synthase (EPSPS) in the shikimate acid pathway, inhibiting the biosynthesis of the aromatic amino acids Trp, Tyr, and Phe. This inhibition causes a reduction in protein and secondary compound biosynthesis (Ye et al., 2001).

Glyphosate-resistant transgenic cotton (Gossypium hirsutum; GRC) containing the CP4-EPSPS gene from Agrobacterium spp. under the cauliflower mosaic virus (CaMV)-35S promoter, exhibits an excellent vegetative tolerance to glyphosate (Pline et al., 2002; Yasuor et al., 2006). However, exposing GRC to glyphosate after the fourth-leaf stage can result in yield reduction due to reduced self pollination (Pline et al., 2003a; Viator et al., 2003, 2004; Yasuor et al., 2006). Morphological changes in the flower organs, production of nonviable pollen grains, and reduced anther dehiscence seem to be the main factors responsible for reduced pollination in glyphosate-treated GRC (Pline et al., 2002, 2003a; Yasuor et al., 2006). The sensitivity of the male reproductive organs to glyphosate in cotton may be explained by reduced expression of the CP4-EPSPS gene in these organs (Chen and Hubmeier, 2001; Pline et al., 2002). Glyphosate-induced partial male sterility in glyphosate-resistant maize (Zea mays) was also attributed to reduction in pollen viability and inhibition of anther dehiscence (Thomas et al., 2004).

The occurrence of anther abnormalities and their severity in glyphosate-treated GRC varied greatly, from partially opened anthers and viable pollen to nondehisced anthers containing nonviable pollen (Pline et al., 2002; Yasuor et al., 2006). Reproduction in plants is highly sensitive to environmental stresses. High temperatures, especially during the night, can have

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This work was supported in part by the Chief Scientist Fund, Ministry of Agriculture, Israel and the Israeli Cotton Board.

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.106.081943.
significant effects on flower development and pollination (Hall, 1992; Ismail and Hall, 1998). However, the effect of high temperatures on glyphosate-induced damage to the male reproductive organs of GRC has not been studied.

As noted above, failure of the anthers to dehisce is one of the main factors leading to reduced pollination in glyphosate-treated plants. The anther dehiscence process consists of the following stages: (1) expansion of the epidermal and endothecium cells and deposition of U or longitudinal secondary cell wall thickening (SWT) structures along the radial and inner tangential walls of the endothecium cells; (2) enzymatic lysis of the septum between two adjacent locules; (3) mechanical rupture of the tapetum; (4) mechanical opening of the stomium by the centripetal force of the highly turgescent epidermis and endothecium cells; and (5) outward bending of the locule walls by the centrifugal force of the dehydrating epidermis and endothecium (Keijzer, 1987; Bonner and Dickinson, 1989; Goldberg et al., 1993; Hegde et al., 1993; Keijzer, 1999; Scott et al., 2004). The SWT allow the endothecium cells to shrink asymmetrically when the anther dries out at anthesis, resulting in breakage of the stomium. Absence or impairment of the stomium breakage will lead to dehiscence failure (Keijzer, 1987; Bonner and Dickinson, 1989; Beals and Goldberg, 1997).

The cell wall is essential for many processes in plant development. The primary cell wall is composed of cellulose microfibrils (MF) embedded in a polysaccharide matrix. Transverse cellulose MF deposition is a well-known phenomenon in expanding cells, such as cortical and vascular cells of stems and roots, restricting isotropic growth and promoting unisotropic cell elongation (Taiz and Zeiger, 1998). Cortical microtubules (CMT), which are part of the cytoskeleton, are complex...
hollow cylinders built from tubulin subunits. CMT are postulated to define the direction and or length of cellulose MF and by this determine the preferential axis of cell growth in plant tissues (Wasteneys, 2004).

Plant hormones can affect CMT organization, for example auxin (Shibaoka, 1994; Takesue and Shibaoka, 1999; Wiesler et al., 2002), gibberellin (Shibaoka, 1994), and brassinosteroid (Bouquin et al., 2003) treatments resulted in a change in the CMT arrangement from longitudinal to transverse orientation. On the other hand, ethylene and abscisic acid induced a transverse to longitudinal reorientation of the CMT (Shibaoka, 1994; Bouquin et al., 2003).

Owing to the inhibition of the shikimic pathway, it is expected that glyphosate will reduce the endogenous indole-3-acetic acid (IAA) level. However, there are contradictory results concerning the effect of glyphosate on IAA level. Westwood and Biesboer (1985) reported that glyphosate caused a decrease in IAA level in leafy spurge (Euphorbia esula) 12 h after treatment. Others reported a 3-fold increase in IAA level in Cyperus esculentus following glyphosate application (Cañal et al., 1987). There are data that glyphosate may induce changes in IAA level by affecting IAA metabolism (Lee, 1982, 1984; Lee and Starratt, 1989; Hoagland, 1990). Glyphosate was also shown to affect auxin transport (Baur, 1979b).

In this study we demonstrate that male sterility in GRC is partially temperature dependent. Further, two main mechanisms seem to be responsible for the inhibition of anther dehiscence: inhibition of the enzymatic lysis of the septum between adjacent locules and a change in the orientation of SWT in the endothecium cells. SWT abnormalities were accompanied by changes in CMT organization. A change in CMT organization following glyphosate treatment was also observed in Arabidopsis (Arabidopsis thaliana) hypocotyls. Glyphosate treatment led to a significant auxin accumulation in the anthers and ectopic application of auxin caused similar SWT abnormalities to those observed following glyphosate application, suggesting that auxin is involved in glyphosate-induced cell wall modifications.

**RESULTS**

**CP4-EPSPS Expression**

Expression of CP4-EPSPS was studied by western- and tissue-blot analyses. Western-blot analysis demonstrated a significantly lower expression of the CP4-EPSPS

Figure 3. Histological sections of anthers from glyphosate-treated and control GRC plants grown at 2 d/night temperature regimes. A and B, Anthers from plants grown at a moderate temperature (28°C/22°C day/night) regime. A, Untreated control and B, glyphosate treated, both showing lysis of the septum and normal appearance of the stomium (ST). C and D, Anthers from plants grown at high temperatures (34°C/28°C). C, Untreated control showed that high temperatures by themselves did not affect anther dehiscence and pollen viability. D, Anthers from glyphosate-treated plants grown at high temperatures demonstrated no septum lysis between two adjacent locules and no stomium formation. E and F, A closer look at the SWT of endothecium cells in anthers from plants grown at high temperatures. E, Untreated control showing that the SWT were deposited along the radial and inner tangential walls (arrows) in the endothecium cells. F, Anthers from glyphosate-treated plants, showing SWT deposited across the endothecium cells (arrows), thus preventing their normal function.
protein in the male reproductive organs compared to the female organs (Fig. 1A). The data showed that neither glyphosate nor temperature treatments had a significant effect on the level of CP4-EPSPS expression in GRC anthers. Tissue-print blot also demonstrated that anther tissues exhibited a low CP4-EPSPS expression level (light staining) compared to the high expression level observed in the ovules, ovaries, and stigma (dark staining; Fig. 1C).

Temperature-Dependent Anther Dehiscence and Pollen Viability

The effect of glyphosate on anther dehiscence and pollen viability was temperature dependent. Anthers developed from glyphosate-treated plants grown at high temperatures ($34^\circ/C$/$28^\circ/C$; Fig. 2E) did not dehisce and no pollen grains were observed outside the anthers during the day of anthesis, whereas anthers from plants grown at moderate temperatures ($28^\circ/C$/$22^\circ/C$) had normal pollen dispersal (Fig. 2C). Pollen grains from glyphosate-treated plants grown at high temperatures were not viable (Fig. 2F), as indicated by Alexander (1969) staining. Their cytoplasm stained blue compared to the red staining in viable pollen grains (Fig. 2B). These pollen grains were also malformed, irregular, and crumpled. In contrast, pollen grains from glyphosate-treated plants grown at moderate temperatures had regular, round turgid shapes, with only about 50% viability. All pollen grains from untreated plants grown under both temperature regimes were viable.

Modification of Anther Dehiscence Mechanisms

The anther-opening mechanism involves several physical and biological processes, one of which is the enzymatic degradation of the septum cells (Keizer, 1987; Goldberg et al., 1993). Histological observations demonstrated that the septum in anthers of untreated and glyphosate-treated plants grown at moderate temperatures underwent enzymatic lysis and disintegrated normally (Fig. 3, A–C). Thus, the stomium in these anthers could be broken at anthesis, allowing anther dehiscence and pollen grain release. However, under high temperature conditions, the septum of glyphosate-treated anthers did not disintegrate (Fig. 3D), so that the stomium could not separate at anthesis, resulting in nondehisced anthers.

The driving force of the anther opening is generated by SWT, which develop on the anticlinal and inner tangential walls of the endothecium cells during flower maturation (Keijzer, 1987). In anthers of glyphosate-treated plants grown at high temperatures, the deposition of the SWT changed from the normal longitudinal orientation to transverse orientation, forming a ladder-shape pattern instead of the normal U-shape pattern (Fig. 3, E and F). In anthers of treated plants grown under moderate temperatures, the orientation of the SWT was solely longitudinal. Similar changes in SWT organization were also observed in nontransgenic cotton treated with a sublethal dose of glyphosate (0.288 kg ae ha$^{-1}$; Fig. 4, A and B).

Changes in SWT Are Accompanied by Modifications in CMT

Since CMT are thought to be involved in the determination of cellulose MF orientation, we studied the effect of glyphosate on microtubule orientation in the endothecium cells. SWT deposition was observed in the endothecium cells only at the late phase of anther development (about 1 week before anthesis). Immunostaining of CMT in these cells was performed on the

![Figure 4. A comparison of cell walls and microtubule arrangement in anther endothecium cells from plants with and without glyphosate treatment. A to D, SWT organization in wild-type (A and B) and GRC (C and D) endothecium cells. A and C, Flowers from untreated plants exhibit SWT in a longitudinal orientation (arrows) in endothecium cells. B and D, SWT of endothecium cells in flowers from glyphosate-treated plants exhibiting a transverse orientation (arrows). E and F, CMT of anther endothecium cells shown in C and D. CMT in anthers from control plants (E) were in a longitudinal organization (small arrows), perpendicular to the epidermis surface (large arrow). F, CMT in anthers from glyphosate-treated plants (F) were in a transverse organization (small arrows), parallel to the epidermis surface (large arrow). Bars = 20 $\mu$m.](https://www.plantphysiol.org/doi/10.1079/PP20060208)
day of anthesis, before pollen dispersal. Confocal microscopy observations showed that in untreated GRC cotton, both the CMT and SWT were organized in longitudinal orientation along the internal tangential and radial walls of the endothecium cells (Fig. 4, C and E). However, in anthers from glyphosate-treated plants, the CMT and SWT were organized in a transverse orientation (Figs. 4, D and F).

To examine whether glyphosate treatment could affect CMT organization in other systems, glyphosate (10 mM) was applied to Arabidopsis seedlings expressing green fluorescent protein (GFP) tubulin (tubulin α 6 [TUA6]). The normal orientation of CMT in hypocotyl epidermal cells of untreated Arabidopsis plants was mostly longitudinal, with a low percentage of oblique, transverse, or random arrangement (Fig. 5, A and B). Glyphosate caused a quick change in the orientation of the CMT. Nine hours after treatment CMT started to reorganize into either oblique or transverse orientation (Fig. 5C). By 24 h after glyphosate treatment, approximately 80% of the hypocotyl epidermal cells showed either oblique or transverse orientation of the CMT and only about 10% were in a longitudinal orientation. The other 10% to 15% of CMT in glyphosate-treated seedlings were randomly arranged, usually radiating from the center of the cell toward the cell cortex.

Figure 5. CMT of GFP tubulin (TUA6). Arabidopsis hypocotyl epidermal cells are responsive to glyphosate. A and B, Images of hypocotyl epidermal cells expressing the GFP tubulin before (A) or 24 h after (B) glyphosate (10 mM) application. Bars = 50 μm. Longitudinal to transverse reorientation of the CMT is observed in treated hypocotyls. C, Quantitative analysis of microtubules orientation in control and glyphosate-treated plants. Angles were determined relative to the longitudinal axis of the cell.

Glyphosate-Induced Accumulation of IAA in Cotton Anthers

Glyphosate (1.44 kg ae ha⁻¹) application at the eight-leaf stage (the beginning of flower bud appearance) resulted in an increase in IAA level in GRC anthers. The IAA level in anthers from glyphosate-treated plants collected 1 d before anthesis was 5-to-10-fold higher compared to the untreated control (Fig. 6A). These data were verified by gas chromatography (GC)-mass spectrometry (MS) analysis of IAA content (data not shown). By following the kinetics of auxin accumulation in anthers of GRC, it was found that accumulation of IAA in developing cotton anthers during the first stages of flower development (until 2 weeks after glyphosate treatment) was quite low, but increased significantly during the last week before anthesis (Fig. 6B). Glyphosate treatment further increased IAA accumulation during the last week before anthesis (14–20 d after treatment). IAA level in this phase was severalfold higher in glyphosate-treated plants compared to the control (Fig. 6B).

2,4-Dichlorophenoxyacetic Acid Inhibited Anther Function

To examine the possibility that high auxin levels in the anthers of glyphosate-treated GRC are involved in
male sterility, plants were treated at the beginning of the flowering stage (10–12 leaf stage) with 2,4-dichlorophenoxyacetic acid (2,4-D; 1 mg L\(^{-1}\)). Glyphosate was applied for comparison at the eight-leaf stage. Male sterile flowers were observed in both treatments (Fig. 7, D–H). Similar to glyphosate treatment, anthers from 2,4-D-treated plants did not dehisce and no pollen dispersal was observed during anthesis. Nonviable pollen (blue staining) was observed in closed anthers from 2,4-D- and glyphosate-treated plants.

**Figure 6.** IAA analysis in cotton anthers. A, IAA accumulation in cotton anthers 1 d before anthesis. B, Kinetics of IAA accumulation during flower development. Glyphosate 1.44 kg ae ha\(^{-1}\) was applied to GRC at the eight-leaf growth stage, and anthers were harvested 5, 10, 14, and 20 d after treatment. NT, Wild type; 0, untreated; Gly, glyphosate treated. Bars represent mean ± se (n = 10).

**Figure 7.** A comparison of flowers from plants treated with glyphosate and 2,4-D. A to C, Untreated control. D to F, Glyphosate treated. G to I, 2,4-D treated. A, D, and G, Binocular images of flowers at anthesis (magnification × 10). B, E, and H, Scanning electron microscope images of anthers taken from flowers shown in the top section, exhibiting similar non-dehisced anthers in the 2,4-D and glyphosate treatments. C, F and I, Light microscopy photographs of pollen grains after staining with Alexander reagent (magnification × 40) demonstrating nonviable pollen (blue staining) in the glyphosate and 2,4-D treatments compared to red staining in the control pollen. Note that the nonviable pollen in 2,4-D-treated flowers (I) had a round shape, unlike the collapsed shape of the glyphosate-treated pollen (F). Bars = 500 μm.
Based on Alexander (1969) staining it can be concluded that 2,4-D induced partial nonviability, but the shape of the pollen was normal.

Similar to anthers from glyphosate-treated plants, 2,4-D treatment caused a change in the organization of the SWT in the endothecium cells, i.e., the SWT were in a transverse orientation instead of the normal longitudinal orientation (Fig. 8).

**Auxin Translocation**

To evaluate whether the increase in IAA level following glyphosate treatment resulted from inhibition of IAA transport out of the anthers, we examined the effect of glyphosate on [3H]1-naphthaleneacetic acid (NAA) translocation in the staminal column and flower peduncle. Glyphosate had no significant effect on [3H]NAA transport in the stamen column (Fig. 9) or flower peduncle (data not shown). In glyphosate-treated flowers, there was some reduction in [3H]NAA in the lower segment and receiver block and a parallel retention of the auxin in the upper segment (Fig. 9), indicating a slight inhibition of auxin transport by the herbicide.

**DISCUSSION**

The observed damage to the male reproductive organs seems to be the result of a low expression of the CP4-EPSPS in these organs (Fig. 1). Similar results were also reported by Pline et al. (2002). Chen and Hubmeier (2001) have also shown a reduced expression of CP4-EPSPS in GRC anther cell layers and in the gametophytes during anther and gametophyte development. Their results, however, focused on the expression level during the early stages of anther development and they did not compare different flower organs. The transgene is expressed under a constitutive promoter, CaMV 35S, which has been shown to exhibit low expression patterns in the pollen grains of Arabidopsis (Wilkinson et al., 1997) and petunia (Petunia hybrida; Benfey and Chua, 1989). Sunilkumar et al. (2002), using GFP as a reported gene, also showed that the CaMV-35S promoter had a low expression in cotton stamens compared to ovules.

It is well documented that the vegetative parts of GRC plants are not affected by glyphosate and no visible damage is observed when the herbicide is applied at various plant growth stages under different growing conditions (Pline et al., 2002; Yasuor et al., 2006). On the other hand, the reproductive organs, particularly the male ones, show a high sensitivity to glyphosate applied after flower bud formation (Pline et al., 2002; Yasuor et al., 2006). In this study, a complete male sterility was observed in glyphosate-treated plants grown at high temperatures, whereas flowers from treated plants grown at moderate temperatures exhibited opened anthers, and only about a 50% reduction in pollen viability (Figs. 2 and 3). It is well documented that male sterility is affected by various environmental factors, particularly a high temperature stress (Ahmed et al., 1992; Peet et al., 1998; Sakata et al., 2000; Erickson and Markhart, 2002; Young et al., 2004) and a low air humidity (Keijzer, 1987; Matsui et al., 1999). High temperatures have been reported to induce male sterility in tomato (Lycopersicon esculentum) and cowpea (Vigna unguiculata) plants due to reduced pollen viability, inhibition of style elongation, and lack of endothecium formation (Rudich et al., 1977; Ahmed et al., 1992). Although high temperatures alone are not sufficient to cause male sterility in cotton, our data suggest that high temperatures increase the sensitivity of cotton male organs to glyphosate. Unlike temperature, water stress did not increase the negative responses of GRC grown in the field or under controlled environment to glyphosate (Pline et al., 2003b).
The data of this study demonstrate that the failure of the glyphosate-treated anthers to dehisce at high temperatures was caused by changes in the deposition of the SWT from vertical to horizontal orientation and inhibition of the septum disintegration (Figs. 3 and 4). It is noteworthy that similar effects of glyphosate were also observed in nontransgenic cotton treated with a sublethal dose (0.208 kg ae ha⁻¹) of the herbicide (Fig. 4). These apparent changes inhibited the mechanical opening of the anthers by the centripetal force of the highly turgescent epidermis and endothecium cells, and therefore no pollen grains could be released at anthesis. To the best of our knowledge this is the first report relating the failure of anthers to dehisce to a change in the orientation of the SWT in the endothecium cells. Previous reports have shown that absence of the endothecium or lack of SWT development may inhibit anther dehiscence (Dawson et al., 1999; Scott et al., 2004). Other modifications that led to male sterility were reported earlier in Arabidopsis mutant ms35 (Dawson et al., 1999). In this mutant, disruption of the lignification of the SWT in the endothecium cells prevented dehiscence and pollen dispersal.

Cell wall structure is closely related to the organization of the CMT (Wasteneys, 2004) and indeed we observed a novel effect of glyphosate on the organization of the CMT array in both cotton (Fig. 4) and Arabidopsis (Fig. 5). The data suggest that glyphosate can affect cell wall structure by changing the orientation of the CMT, a phenomenon that might not be related to the inhibition of the shikimic acid pathway. IAA accumulated in both control and glyphosate-treated anthers during the last stages of flower development, but its level in treated anthers was severalfold higher than that in the control (Fig. 6). A recent report also indicates that anthers are major sites of high concentrations of IAA that retard the development of neighboring floral organs (Aloni et al., 2006). Since IAA levels during the last stage of anther development were significantly higher in glyphosate-treated plants, it is quite possible that this increase was responsible for the cellular changes induced by the herbicide. This assumption is supported by several types of evidence: (1) 2,4-D treatment resulted in male sterility, which was characterized by SWT reorientation in the endothecium cells similar to that induced by glyphosate (Figs. 4 and 9); (2) Anther-specific expression of the rolB gene of Agrobacterium rhizogenes demonstrated that IAA plays an important role in anther development (Spena et al., 1992; Cecchetti et al., 2004). Increased levels of IAA caused by the transgene altered anther development and delayed anther dehiscence; and (3) plant hormones, including IAA, can affect CMT orientation and hence the orientation of cellulose MF in the cell wall (Takesue and Shibaoaka, 1999; Bouquin et al., 2003; Wasteneys and Collings, 2004). At present, it is not clear whether IAA is also involved in CMT reorientation in Arabidopsis. The reorientation in Arabidopsis occurred very rapidly following glyphosate application compared to cotton, and preliminary analysis of IAA did not show significant differences between the control and glyphosate-treated plants.

The increased level of IAA in glyphosate-treated anthers is surprising, since both precursors of IAA, Trp and indole, are synthesized via the shikimic acid pathway (Woodward and Bartel, 2005). The low expression of the transgene in the anthers implies that the shikimic acid biosynthesis in these organs is inhibited. An increased IAA level in glyphosate-treated plants was reported before (Cañal et al., 1987). In addition, glyphosate treatment was shown to mimic responses that are normally associated with auxin, such as release of lateral buds from apical dominance (Lee, 1984) and increased tillering (Baur, 1979a). In contrast, Lee (1982) reported a reduction in IAA level following glyphosate treatment. At present we do not have a good explanation for the significant accumulation of IAA following glyphosate treatment. One possible explanation is the inhibition of IAA transport from the anthers to other floral organs. Baur (1979b) reported that glyphosate decreased the velocity of polar IAA transport in cotton stem segments. However, we did not observe a significant decrease in IAA transport in the staminal column following glyphosate treatment (Fig. 9), indicating that inhibition of IAA transport was not the causal agent for IAA accumulation.

Taken together our data demonstrate that male sterility observed in glyphosate-treated cotton plants is exasperated by high temperature and involves modifications in CMT and SWT orientation in the endothecium cell layer, which are mediated by increased accumulation of IAA in the anthers.

MATERIALS AND METHODS

Plant Material

Cotton (Gossypium hirsutum) plants were grown in 10 L peatsandy soil (1:1 v/v) in a greenhouse until the eight-leaf stage. GRC (cv DP5415RR) and nontransgenic isogenic line (cv DP5415) plants were sprayed with glyphosate (1.44 and 0.288 kg ae ha⁻¹, respectively) using a commercial formulation of glyphosate (Roundup Ultra, 360 g ae L⁻¹, Monsanto) and transferred to a phytotron for further growth at two different day/night temperature regimes (28°C/22°C and 34°C/28°C day/night) and 16 h daylight. GRC plants were treated with 2,4-D (1 mg L⁻¹ + 0.1% BBS surfactant) at the beginning of flowering and the plants were grown at the high temperature regime.

Arabidopsis (Arabidopsis thaliana) expressing GFP TUA6, kindly provided by Dr. Takashi Hashimoto (Nara Institute of Science and Technology, Nara, Japan), was sown in Murashige and Skoog agar plates (Murashige and Skoog, 1962) and grown at 23°C and 16 h light. Ten-day-old seedlings were dipped in 10 mM glyphosate for 5 s and transferred to new Murashige and Skoog agar plates for further development. GFP microtubules were monitored in hypocotyl epidermal cells before glyphosate treatment and during 3 h after treatment.

CP4-EPSPS Protein Analysis, Western Blot

Plants were homogenized in extraction buffer (9 M urea, 75 mM Tris buffer [pH 6.8], 4.5% [v/v] SDS, and 7.5% [v/v] β-mercaptoethanol [1:4, v:v]). After homogenization, the extracts were kept at room temperature for 30 min, boiled for 5 min, and then centrifuged at 10,000g for 10 min. The supernatant was brought to a final concentration of 10% glycine. Samples were either used
immediately or stored at −20°C for further analysis. Total protein concentration in the extracts was determined according to Bradford (1976). Equal amounts of protein were separated on 12% SDS polyacrylamide gel and transferred onto nitrocellulose filters. The CP4-EPSPS protein was detected with a polyclonal antiserum raised against CP4-EPSPS (1:10,000; Monsanto).

**CP4-EPSPS Protein Analysis, Tissue-Print Blot**

Flower buds were cut in half using a razor blade and the cut surface was blotted on a nitrocellulose membrane for 5 s. The membranes were incubated for 20 min at 80°C to eliminate endogenous alkaline phosphates (AP) activity. The CP4-EPSPS protein was detected with a polyclonal antiserum raised against CP4-EPSPS (1:5,000; Monsanto). Visualization of secondary antibodies conjugated to AP was performed according to ProtBlot II AP system with a nitrocellulose filters. The CP4-EPSPS protein was detected with a polyclonal antiserum raised against CP4-EPSPS (1:10,000; Monsanto). Visualization of secondary antibodies conjugated to AP was performed according to ProtBlot II AP system with a stabilized substrate (Promega).

**Light Microscopy**

For light microscopy, flower buds were collected 1 d before anthesis. The buds were fixed in formaldehyde-glacial acetic acid-ethyl alcohol 70% (5:5:40, v/v/v) and stored at 4°C. The buds were gradually dehydrated in an ethyl alcohol-xylene series, stained with 1% safranine (Sigma), and embedded in paraffin wax blocks. Cross sections, 8-μm thick, were prepared using a microtome (Leica RM 2165, Leica Instruments GmbH) and observed under a light microscope (Olympus model BH-2). Pollen viability was determined at anthesis according to Alexander (1969).

**Cytoskeleton Immunostaining**

Immunostaining of CMT was performed according to Lahav et al. (2004) with slight changes. Anthers collected on the day of anthesis, before pollen dispersal, were dissected in the stomium region and fixed for 1.5 h in PME buffer (50 mM PIPES, pH 6.9, 5 mM EGTA, and 2 mM MgSO4) containing 3% paraformaldehyde. The anthers were then washed 3 × 30 min in phosphate-buffered saline (PBS) and incubated in PME buffer containing 1% cellulase, 0.1% pectinase, and 1% Triton X-100 for 45 min, followed by 3 × 30 min washings with PBS. Membrane permeability was obtained by the freeze-shattering method (Wasteney et al., 1997). Blocking was performed overnight at 4°C in PBS containing 1% bovine serum albumin and 0.05% Triton X-100. Incubation with the antibodies was for 2 h for the first antibody anti-α-tubulin (Sigma, DPM19026; 1:100 in blocking buffer) and for additional 1 h for the second antibody goat anti-mouse conjugated to Cy3 (Jackson 115-165-072) at room temperature, followed by 3 × 30 min washings with PBS. The anthers were then mounted in Elvanol under a coverslip.

**Fluorescent Microscopy and Image Analysis**

Live cell imaging was carried out by mounting intact Arabidopsis hypocotyls under a cover glass, and acquiring serial (XYZ) optic sections and projection. All microscopic observations and image acquisitions were performed using the OLYMPUS IX 81 inverted laser-scanning confocal microscope (FLUOVIEW 500) equipped with a 543 nm helium-neon laser and 60 × 1.4 NA PlanApo oil immersion objective. Cy3 was excited by 543 nm light and the emission was collected through an BA 560 IF filter. Confocal optical sections were obtained at 0.2 μm increments. Three-dimensional images were obtained using the FLUOVIEW 500 software supplied with the confocal laser-scanning microscope. The transmitted light images were obtained using Nomarski differential interference contrast. Anthers morphology was examined directly, using a JEOL scanning electron microscope (model JSM-5410LV).

**IAA Quantification**

IAA was routinely determined by radioimmunoassay (RIA) and the data were verified by GC-MS analysis. IAA was extracted and partially purified according to Thompson et al. (1981) with some modifications. Five-hundred milligrams of anther tissue were homogenized with a mortar and pestle in 5 mL of 80% methanol containing 100 mM ammonium acetate and 45 μM butylated hydroxytoluene. Homogenization was performed for 1 min in an ice bath. The homogenized samples were kept in the dark at 4°C. After 30 min the samples were vigorously mixed, centrifuged at 10,000 rpm for 15 min, and the supernatant was saved. The pellet was resuspended in 5 mL of distilled water and centrifuged as above. Both supernatants were pooled and IAA was subjected to three open-column liquid chromatography steps. The extract was loaded onto polyvinylpolypyrrolidone column and IAA was eluted with 10 mM ammonium acetate. The elute was then loaded directly onto a DEAE-Sephadex column in the acetate form equilibrated with the same solution. The IAA was eluted from the DEAE-Sephadex column with 1 M acetic acid and applied to a C18 Sep-Pak column. The column was washed with distilled water to remove acetic acid and IAA was eluted with methanol. The methanol solution containing the IAA was evaporated to a small volume and dried under a stream of nitrogen. The use of labeled IAA as internal standard indicated that recovery of IAA was about 60% to 70%. The IAA was dissolved in 100 μL methanol and methylated with 900 μL diazomethane for 20 min with gentle shaking. The methanol and diazomethane were evaporated under N2 and redissolved in 50 μL of methanol and 950 μL RIA buffer. IAA was quantified by RIA according to Weiler (1981).

For GC-MS analysis IAA was extracted and purified from 100 mg anthers according to Roessner-Tunali et al. (2003) and GC-MS analysis was performed by Dr. Joachim Kopka, Max-Planck Institute, Golm, Germany.

**Auxin Transport**

The basal side of 10-mm sections of flower peduncles and staminal columns were placed each on 1.5% agar discs (9 mm in diameter and 3 mm thick). The discs were placed on a Parafilm layer in petri dishes, and the dishes were placed in a humid container. Three microliters of water containing 200,000 dpm of [3H]NAA (specific activity 10 mCi/mmol) were applied to the acropetal surface of the segments. Auxin transport was carried out at 25°C for 6 h. At the end of the incubation, the sections were cut in half and each section and the receiver agar disc were transferred to separate vials containing 4 mL of scintillation solution. [3H]NAA was extracted overnight with constant shaking at room temperature and counted by means of a liquid scintillation counter.

**ACKNOWLEDGMENTS**

We thank Prof. Eli Zamski for his help during the study, Mr. Elik Abiri for his continuous support, Delta & Pine (USA) for providing the cotton seeds, and Monsanto (St. Louis, Missouri) for the anti-CP4-EPSPS antibodies. The help of Agan Chemicals (Israel) is also appreciated.

Received April 12, 2006; revised May 30, 2006; accepted May 30, 2006; published June 9, 2006.

**LITERATURE CITED**

Baur JR (1979a) Reduction of glyphosate-induced tillering in sorghum (Sorghum bicolor) by several chemicals. Weed Sci 27: 69–73