Formation of root silica aggregates in sorghum is an active process of the endodermis

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Supplementary Protocols S1

Raman Analysis

Comparing silica aggregates forming ex planta vs in planta

We used Raman spectroscopy to test the effect of ex planta growth of root segments on the ITCW composition, in comparison to ITCW forming in hydroponically grown seedlings, herein in planta growth (Fig. S1). Si supplementation did not change the mean signal significantly, as suggested by the overlapping spectral standard deviations of ITCW that developed with (Si+) or without (Si-) the addition of silicic acid (Fig. S1a). To compare the effect of the growing system on the ITCW composition we subtracted the mean ITCW spectrum of Si+ ex planta samples from the ITCW mean spectrum of Si+ in planta samples (Fig. S1b). Both spectra were normalized against the crystalline cellulose band at 380 cm⁻¹, and therefore cellulose peaks are assumed to vanish under this operation. The positive difference spectrum indicates that in relation to cellulose, the in planta ITCW contains more matrix polymers, including hemicelluloses and lignin, as compared with the ex planta ITCW. We further subtracted the mean Si+ ex planta spectrum from each of the spectra measured both in
*planta* and *ex planta* and calculated the standard deviation of the mean difference spectra (Fig. S1c). Since the deviations do not overlap we concluded that the reduction in cell wall matrix polymers under *ex planta* growth is significant.

**Figure S1.** Raman spectra and spectral differences of the ITCWs of primary root endodermis after cultivation in Si+ or Si- medium. (a) Mean spectra ± their standard deviation (SD) of the ITCWs recorded after either a six-day hydroponic cultivation of intact plants (*in planta*), or a three-day cultivation of detached root segments (*ex planta*). No significant differences are detected between spectra of cell wall grown with Si (Si+, gray) or without Si (Si-, red). Inset – comparison of the mean spectra. (b) Difference of the mean spectrum of Si+ *ex planta* treatment from the mean hydroponic Si+ spectrum (yellow). Coloured areas indicate the standard deviations of the hydroponics (grey) and *ex planta* (red) treatments, after the subtraction of the mean Si+ *ex planta* spectrum. One can appreciate that more polysaccharides and aromatic materials in relation to cellulose are deposited in the hydroponics samples by the residual positive yellow spectrum. Spectra were normalized to the cellulose peak at 380 cm⁻¹. For peak assignment see Table S1.

**Testing the effect of specific matrix polymers on silica aggregates formation**

We aimed to test whether silica aggregate formation is linked to specific cell wall components. We first applied the exocytosis inhibitor brefeldin A to inhibit hemicellulose delivery to the wall. Raman analyses of the endodermis ITCWs showed that the treatments reduced peaks attributable to ferulic acid, arabinoxylans, and phenolic compounds in general (Fig. S2, Table S1).
Figure S2. Raman difference spectra of the endodermal ITCW of root segments cultivated ex planta in the presence of exocytosis inhibitor Brefeldin A. Endodermal ITCWs were sampled after 72 h ex planta cultivation in Si+ 1 mM medium supplied with (a) 1 mM, (b) 5 mM, and (c) 10 mM brefeldin A in 0.01 % (v/v) ethanol. In red is the standard deviation of the spectra collected from ITCW of root segments grown under control conditions (a Si+ solution at 1mM Si concentration). In grey is the standard deviation of the spectral difference between the brefeldin A treated and untreated root segments. In yellow is the mean spectral difference. Spectra were normalized to the 380 cm$^{-1}$ cellulose peak. With the increasing concentration of brefeldin A a significant reduction all spectral features is observed.

The addition of exogenous ferulic acid (FA) reduced the deposition of phenolic compounds (Fig. S3). This may indicate that the exogenous FA acted as a reactive oxygen species scavenger rather than cell wall precursor (Mathew and Abraham 2004). Both brefeldin A and ferulic acid affected negatively the formation of silica aggregates, reducing their Si:C ratios (Fig. S4).
Figure S3. Raman difference spectra of the endodermal ITCW of root segments cultivated \textit{ex planta} in the presence of ferulic acid. Endodermal ITCWs were sampled after 72 h \textit{ex planta} cultivation in Si+ 1 mM medium supplied with (a) 0.05 mM and (b) 0.2 mM ferulic acid. In red is the standard deviation of the spectra collected from ITCW of root segments grown under control conditions (Si+ solution at 1 mM Si concentration). In grey is the standard deviation of the spectral difference between the ferulic acid treated and untreated root segments. In yellow is the mean spectral difference. Spectra were normalized to the 380 cm\(^{-1}\) cellulose peak. Some reduction in the peaks of lignin and aromatic substances (1600 and 1630 cm\(^{-1}\)) are observed under the higher ferulic acid concentration.

Figure S4. Aggregate Si:C ratios in segments grown \textit{ex planta} in Si+ 1 mM (control) media supplied with exocytosis inhibitor brefeldin A (BFA) or ferulic acid (FA). Different letters indicate statistically significant differences at \( p \leq 0.05 \).

To inspect the relationship between lignin and silica formation we aimed to change the balance of reactive oxygen species in the root segments cultivated \textit{ex planta}. As the deposition of
phenolic compounds is frequently mediated by class III peroxidases, we added to the cultivation medium salicylhydroxamic acid (SHAM), a peroxidase inhibitor. We detected a notable reduction in the abundance of phenolic compounds in the ITCWs (negative difference signal at 1600-1700 cm$^{-1}$, characteristic to phenolics, Fig. S5, Table S1).

Figure S5. Raman difference spectra of the endodermal ITCW of root segments cultivated ex planta in the presence of SHAM, a peroxidase activity inhibitor. Endodermal ITCWs were sampled after 72 h ex planta cultivation in Si+ 1mM medium supplied with (a) 0.2 mM or (b) 2 mM SHAM. In red is the standard deviation of the spectra collected from ITCW of root segments grown under control conditions (Si+ solution at 1 mM Si concentration). In grey is the standard deviation of the spectral difference between the SHAM treated and untreated root segments. In yellow is the mean spectral difference (SHAM-control treatments). Spectra were normalized to the 380 cm$^{-1}$ cellulose peak.

We then aimed to decrease lignification by scavenging reactive oxygen species in the cell walls of root segments. We applied the H$_2$O$_2$ scavengers KI and ascorbic acid. Raman difference spectra indicated a decrease in the deposition of wall matrix polymers, predominantly aromatic molecules, under these treatments, in comparison to control conditions (Fig. S6, S7, Table S1).
Figure S6. Raman difference spectra of the endodermal ITCW of root segments cultivated ex planta in the presence of KI, a H$_2$O$_2$ scavenger. Endodermal ITCWs were sampled after 72 h ex planta cultivation in Si+ 1 mM medium supplied with (a) 0.5 mM or (b) 5 mM KI. In red is the standard deviation of the spectra collected from ITCW of root segments grown under control conditions (Si+ solution at 1 mM Si concentration). In grey is the standard deviation of the spectral difference between the KI treated and untreated root segments. In yellow is the mean spectral difference. Spectra were normalized to the 380 cm$^{-1}$ cellulose peak. One can note that at the lower concentration there are no significant differences between the treatment and the control samples. However, under higher KI treatment, there is a statistically significant decrease in the lignin and aromatic peaks at 1600 and 1630 cm$^{-1}$.

Figure S7. Raman difference spectra of the endodermal ITCW of root segments cultivated ex planta in the presence of ascorbic acid, a H$_2$O$_2$ scavenger. Endodermal ITCWs were sampled after 72 h ex planta cultivation in Si+ 1 mM medium supplied with (a) 1 mM or (b) 5 mM ascorbic acid. In red is the standard deviation of the spectra collected from ITCW of root segments grown under control conditions (Si+ solution at 1 mM Si concentration). In grey is the standard deviation of the spectral difference between the ascorbic acid treated and untreated root segments. In yellow is the mean spectral difference. Spectra were normalized to the 380 cm$^{-1}$ cellulose peak.
In agreement with these results, addition of H$_2$O$_2$ to the cultivation media induced a significant increase in the abundance of phenolic compounds in the ITCWs, as indicated by the positive areas in the difference spectra (Fig. S8). We evaluated the correlation of the matrix composition to silica aggregate formation. A substantial decrease in EDX Si:C ratio was observed under all treatments (Fig. S9).

Figure S8. Raman difference spectra of the endodermal ITCW of root segments cultivated ex planta in the presence of H$_2$O$_2$. Endodermal ITCWs were sampled after 72 h ex planta cultivation in Si+ 1mM medium supplied with (a) 1 mM H$_2$O$_2$ or (b) a pretreatment by 5 mM H$_2$O$_2$. In red is the standard deviation of the spectra collected from ITCW of root segments grown under control conditions (Si+ solution at 1 mM Si concentration). In grey is the standard deviation of the spectral difference between the H$_2$O$_2$ treated (a) or pretreated (b) and untreated root segments. In yellow is the mean spectral difference. Spectra were normalized to the 380 cm$^{-1}$ cellulose peak. A significant increase in aromatic and ferulic acid peaks is observed at 1173, 1600 and 1630 cm$^{-1}$. 

Figure S9. Aggregate Si:C ratios in segments grown ex planta in Si+ media supplied with reagents affecting the peroxidase-mediated oxidative coupling reactions. All media contained 1 mM Si in addition to the following treatments: no additional treatment (control); peroxidase inhibitor [SHAM at 0.2 and 2 mM]; H$_2$O$_2$ scavengers [ascorbic acid (asc) at 1 and 5 mM, and KI at 0.5 and 5 mM]; H$_2$O$_2$ [at 1 mM]. Additionally, segments were pretreated with H$_2$O$_2$ (1 mM) prior to the ex planta cultivation conducted without any additional treatment (H$_2$O$_2$ pre). Different letters indicate statistically significant differences at p ≤ 0.05.

Raman microspectroscopy methodology

Segments of sorghum root seedlings were cultivated ex planta as described in the main text. Treatments applied to the segments are detailed in Table 1. Cross-sections were prepared from the root segments fixed in FAA either before the ex planta cultivation, after the cultivation, or at both time points. Several cross-sections from each root segment were placed on microscope slides, washed three times and mounted in distilled water, covered with cover slips, and sealed with nail polish to avoid water evaporation. Raman spectra were collected with an InVia spectrometer (Renishaw, UK) equipped with 532 nm laser, utilizing WIRE3.2 software (Renishaw, UK). Measurements were performed with 100 kWcm$^{-2}$ laser intensity, 0.1 s acquisition time and 150 accumulations per spectrum. At least 5 spectra from each root were collected, using at least three different roots per treatment. Collected spectra were smoothed (Savitzky-Golay algorithm, 9-point interval, polynomial order 4) and baseline corrected (adaptive baseline correction, coarseness 10%) using Spectragryph 1.0.7 (F. Menges "Spectragryph - optical spectroscopy software", Version 1.0.7, 2017, http://www.effemm2.de/spectragryph/).
Table S1. Raman assignments for the identified peaks in the endodermal cell wall of root segments.

| peak position (cm⁻¹) | assignment | group     | component                      | reference |
|----------------------|------------|-----------|--------------------------------|-----------|
| 350                  | C-O-C deformation | polysaccharides | pectin/hemicellulose | 6         |
| 380                  | δ (CCC) ring; CCO stretching | polysaccharides | crystalline cellulose | 1,3,6,7   |
| 470-515              | coupled modes of heavy atoms, C-C and C–O stretching | polysaccharides | hemicellulose | 7         |
| 494                  | ν (COC) glycosidic bond | polysaccharides | AX | 5,6,7,15 |
| 520                  | skeletal deformation | polysaccharides | | 1,3       |
| 643                  | in-plane ring and skeletal deformation | phenolics | H-lignin | 8         |
| 800-815              | symmetric Si–O–Si stretching | silica | | 12,13    |
| 817                  | ring γ C-OH | polysaccharides | pectin | 20        |
| 898                  | (C-O-C) skeletal | polysaccharides | | 18        |
| 930                  | ν(CO)ring, ν(CC)ring, β(CCH) | polysaccharides | X | 21        |
| 983                  | C–O stretch and ring mode region | polysaccharides | X | 1,3,5,7 |
| 985                  | Si–OH | polysaccharides | | 4,14      |
| 1093                 | ν C-O-C glycosidic symmetric | polysaccharides | | 7,15      |
| 1121                 | ν C-O-C glycosidic asymmetric | polysaccharides | | 7,15      |
| 1141                 | lignin methoxy vibrations, aromatic CCH bend | phenolics | S-lignin | 9,16,19   |
| 1168                 | ring plane deformation CH, C–O(H) stretching | phenolics | FA | 7,11,17   |
| 1200                 | lignin-hydroxyl vibrations | phenolics | lignin | 10        |
| 1213                 | lignin methoxy vibrations | phenolics | lignin | 10        |
| 1270                 | ring deformation, aryl-OCH₃ and aryl-OH in-plane bending | phenolics | H-lignin | 19        |
| 1314                 | ring deformation and C-O stretch | phenolics | G-lignin, FA | 9,19      |
| 1334                 | symmetric COC stretches of two (O)CH₃ groups | phenolics | S-lignin | 9,19      |
| 1378                 | lignin methoxy deformation, methyl bending, aromatic skeletal vibrations | phenolics | lignin | 10        |
| 1424                 | lignin methoxy deformation, methyl bending, aromatic skeletal vibrations | phenolics | | 10        |
| 1456                 | δ CH₂; δ COH; CH/CH₂ wagging: | polysaccharides | hemicellulose | 1,3,6,7,15 |
| 1508                 | Aryl ring stretch, asymmetric aromatic C–C stretch | phenolics | lignin | 9         |
| 1575                 | | phenolics | G/H-lignin | 8         |
| 1618                 | ν C=C aromatic | phenolics | coniferyl/sinapyl aldehyde | 2,16      |
| 1630                 | ν C=C of aryl sidechain | phenolics | FA, coniferyl aldehyde, sinapyl aldehyde | 9,10      |
| 1655-1660            | C≡C stretch | phenolics | CA, sinapyl alcohol | 9,10      |
| 1700                 | Ester C≡O stretches | lipids, polysaccharides | hemicellulose | 18,22     |

X – xylan, AX- arabinoxylan, FA- ferulic acid, CA-coniferyl alcohol, [1] Agarwal and Ralph (1997); [2] Agarwal et al. (2011); [3] Agarwal (2014); [4] Aguiar et al. (2009); [5] Barron et al. (2006); [6] Gierlinger et al. (2008); [7] Himmelsbach and Akin (1998); [8] Larsen and Barsberg (2010); [9] Lupoi and Smith (2012); [10] Lupoi et al. (2015); [11] Ma et al. (2014); [12] Marsich et al. (2009); [13] McMillan (1984); [14] McMillan and Remmel Jr. (1986); [15] Piot et al. (2001); [16] Prats Mateu et al. (2016); [17] Ram et al. (2003); [18] Schulz and Baranska (2007); [19] Sun et al. (2012); [20] Synytsya et al. (2003); [21] Wiercigroch et al. (2017); [22] Wu et al. (2011)
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