Impact of Atmospheric H_2S, Salinity and Anoxia on Sulfur Metabolism in *Zea mays*

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Abstract Plants in coastal salt marshes have to deal with salinity, anoxia and excessive reduced sulfur at the same time. Sulfur metabolism is presumed to have significance in plant stress-tolerance. In order to obtain more insight into the physiological significance of sulfur metabolism in plant responses to multiple abiotic stress factors, the glycophyte maize (*Zea mays*) was exposed to atmospheric H_2S, salinity and anoxia. Maize seedlings appeared to be rather unsusceptible for the potentially toxic effects of these stressors. A 7-day exposure to 0.25 μl l^{-1} H_2S and/or anoxia (anoxic root conditions) slightly enhanced plant biomass production, whereas it was not affected upon exposure to 100 mM NaCl. A simultaneous exposure of plants to salinity with H_2S and/or anoxia resulted in a decreased biomass production. The total sulfur content of the shoot and root was hardly affected by H_2S exposure, whereas it was strongly decreased upon anoxia. The total sulfur content of the shoot was decreased upon exposure to salinity. The decreases in total sulfur content could be predominantly ascribed to a decrease in the sulfate content. H_2S exposure only resulted in an enhanced water-soluble non-protein thiol content in shoots, whereas it was not affected by salinity and anoxia. Only a simultaneous exposure of plants to H_2S, salinity and/or anoxia resulted in an enhanced water-soluble non-protein thiol content of the root. Anoxia and salinity exposure induced aerenchyma formation in the root, and the increased root thiol contents might be the result of the direct diffusion of atmospheric H_2S via the stomata through the aerenchyma and subsequent metabolism in the root.

In nature plants are often exposed to multiple abiotic stress factors. For instance, plants in coastal salt marshes not only have to deal with salinity, but also with anoxia and excessive reduced sulfur. Anoxia (*viz*. anoxic root conditions) limits

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root respiration and carbohydrates are then broken down via fermentative pathways to yield at least some ATP, which often is insufficient to support optimal plant growth (Yamauchi et al. 2013). An adaptation to anoxia exposure is the formation of aerenchyma: spongy tissue that consists of air spaces and channels in the leaves, stems and roots (Yamauchi et al. 2013). Aerenchyma facilitates an enhanced O_2 diffusion from the shoot to the root and this enables root respiration at anoxic conditions (Yamauchi et al. 2013). Exposure to salinity may negatively affect metabolism and plant growth by affecting the water balance and by causing an accumulation of the toxic cation sodium in the cytosol (Grattan and Grieve 1999; Parida and Das 2005). Exposure to sulfide in soil and atmosphere may also be harmful (Beauchamp et al. 1984; De Kok et al. 2002). Hydrogen sulfide is a potentially phytotoxic gas, since it may react with metalloenzymes (*viz*. cytochrome oxidase; Beauchamp et al. 1984; De Kok et al. 2002). However, at low levels foliarly absorbed H_2S may be metabolized and replace sulfate taken up by the root as sulfur source for growth (De Kok et al. 1997, 1998; Hawkesford and De Kok 2006).

It is presumed that sulfur metabolites may fulfill a role in the tolerance of plants to abiotic and biotic stress (Bloem et al. 2014). A variety of organic sulfur compounds are presumed to have stress-protective functions (Rausch and Wachter 2005). For instance, glutathione and derived metabolites are thought to have diverse functions in stress-protection (Tausz et al. 2004; Noctor et al. 2012). In order to obtain more insight into the physiological significance of sulfur metabolism in plant responses to multiple abiotic stress factors, the glycophyte maize (*Zea mays*) was exposed to atmospheric H_2S, salinity and anoxia.

Maize (*Zea mays* subsp. *mays*, cv. Ricardinio, Van der Wal, Hoogeveen, The Netherlands) was germinated on moistened filter paper at 21 °C in the dark for 2 days. Subsequently, the seedlings were transferred to 15 l containers containing tap water in a climate-controlled room at a day/night temperature of 21 °C/18 °C (±1 °C), a relative humidity of 60–70% and a photoperiod of 14 h at a photon fluence rate of 300 ± 20 μmol m^{-2} s^{-1} (within the 400–700 nm range) at plant height, supplied by Philips GreenPower LED (deep red/white 120) production modules. After 7 days the seedlings were transferred to 13 l containers (ten plant sets per container, six plants per set) containing an aerated (oxic) or non-aerated (anoxic) 25% Hoagland nutrient solution (pH 7.0; for composition see Koralewska et al. 2007; the latter nutrient solution was also daily flushed with N_2 for 10 min). Seedlings were exposed to 0.25 μl l^{-1} H_2S and/or 100 mM NaCl for 7 days. The containers with seedlings were placed in 150 l cylindrical stainless steel cabinets (0.6 m diameter) with a polymethyl methacrylate top. Air exchange inside the cabinets was 40 l min^{-1} and the air inside the cabinets was stirred continuously by a ventilator. Day/night temperatures were 22 °C/19 °C (±1 °C), relative humidity was 20–40% and the photoperiod was 14 h at a photon fluence rate of 340 ± 20 μmol m^{-2} s^{-1} (within the 400–700 nm range) at plant height, supplied by Philips GreenPower LED (deep red/white 120) production modules. Temperature was controlled by adjusting the temperature of the cabinet wall. Temperature, relative humidity and photon fluence rate at plant height were monitored using data loggers (Hobo type U12, Onset Computer Corporation, Bourne, MA, USA). For

atmospheric H_2S exposure, pressurized H_2S gas diluted with N_2 gas (1 ml l^{-1}) was injected into the incoming air stream and the concentration in the cabinet was adjusted to the desired concentration of 0.25 µl l^{-1} using mass flow controllers (ASM, Bilthoven, The Netherlands). H_2S concentrations in the cabinets were monitored by an SO_2 analyzer (model 9850) equipped with a H_2S converter (model 8770, Monitor Labs, Measurement Controls Corporation, Englewood, CO, USA). The lids of the containers and plant sets were sealed in order to prevent the absorption of atmospheric H_2S by the solution. On the day before harvest, chlorophyll *a* fluorescence (F_v/F_m ratio) of leaves was measured by using a modulated fluorometer in the morning after a dark-adaptation of at least 1 h (PAM 2000, Walz GmbH, Effeltrich, Germany). Moreover, roots were examined for the presence of aerenchyma under a light microscope. At the day of harvest, shoots and roots of plants were separated and fresh weight was determined. Biomass production was calculated by subtracting final fresh weight from initial fresh weight. For determination of the dry matter content, plants were dried at 80 °C for 24 h. For chlorophyll analysis, pigments were extracted from frozen shoots as described by Shahbaz et al. (2010), and the chlorophyll a and b content were determined according to Lichtenthaler (1987). Water-soluble non-protein thiols were extracted from freshly harvested plants (Shahbaz et al. 2010) and the total water-soluble non-protein thiol content was determined colorimetrically according to De Kok et al. (1988). For determination of total sulfur, sulfate and the mineral nutrient composition, dried shoots and roots were pulverized by using a Retsch Mixer-Mill (type MM2, Haan, Germany). The total sulfur and sulfate content were determined as described by Aghajanzadeh et al. (2016). The mineral nutrient composition was analyzed by inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma atomic emission spectrometry (ICP-AES) as described by Reich et al. (2016b). Data was statistically analyzed by an unpaired Student's t-test at $P \leq 0.01$.

Maize appeared to be not very susceptible to the toxic effects of H_2S and a 7-day exposure to 0.25 µl l^{-1} H_2S resulted in a slight increase in the plant biomass production, accompanied with a slight decrease in the dry matter content of the shoot and root, whereas the shoot to root ratio was not affected (Table 1). A similar increase in biomass production at low levels of atmospheric H_2S has been observed in some other plant species (Thompson et al. 1979; Durenkamp et al. 2007). The chlorophyll content of the shoot, the chlorophyll a/b ratio and chlorophyll *a* fluorescence, the latter represents the quantum yield of photosystem II, were not affected (Table 1). Moreover, the mineral composition of both shoot and root were hardly affected upon H_2S exposure (Table 3). H_2S exposure did also not affect the total sulfur and sulfate content of both shoot and root (Tables 1 and 3), indicating a down-regulation of the sulfate uptake by the root. The latter was supported by the observation that H_2S exposure resulted in a partial but significant decrease in the sulfate uptake capacity of the root (data not shown). H_2S exposure resulted in an increase in the content of water-soluble non-protein thiols (presumably cysteine and glutathione) in the shoot, whereas that in the root remained unaffected (Table 1). The latter data were similar to observations with other plant species (De Kok et al. 1997, 1998).

Table 1 The impact of atmospheric H_2S and NaCl salinity on biomass production, chlorophyll content, chlorophyll *a* fluorescence and sulfur metabolite content of maize

	Control	H_2S	NaCl	H_2S + NaCl
Plant				
Biomass production	4.06 ± 0.88b	5.80 ± 0.93c	3.72 ± 0.76b	2.92 ± 0.54a
Shoot/root ratio	1.01 ± 0.13a	1.09 ± 0.13a	1.26 ± 0.11b	1.15 ± 0.15ab
Shoot				
DMC	9.6 ± 0.4b	8.8 ± 0.3a	10.9 ± 0.6c	11.2 ± 0.4c
Chl a + b	0.67 ± 0.09a	0.79 ± 0.13a	1.11 ± 0.24b	1.26 ± 0.30b
Chl a/b	2.6 ± 0.4a	2.5 ± 0.5a	2.7 ± 0.2a	2.6 ± 0.1a
F_v/F_m	0.75 ± 0.05a	0.74 ± 0.06a	0.77 ± 0.04a	0.76 ± 0.04a
Thiols	0.45 ± 0.10a	0.59 ± 0.03b	0.48 ± 0.06a	0.75 ± 0.03c
Sulfate	91 ± 6b	61 ± 15ab	52 ± 11a	64 ± 18ab
Total sulfur	168 ± 10b	157 ± 24ab	131 ± 12a	149 ± 7ab
Root				
DMC	7.4 ± 0.3b	6.7 ± 0.3a	7.8 ± 0.4c	8.1 ± 0.6c
Thiols	0.46 ± 0.06ab	0.41 ± 0.04a	0.40 ± 0.05a	0.51 ± 0.03b
Sulfate	81 ± 21ab	89 ± 9ab	108 ± 6b	91 ± 6a
Total sulfur	152 ± 18a	156 ± 22a	166 ± 11a	172 ± 9a
Aerenchyma	Absent	Absent	Present	Present

Plants were exposed to 0.25 μl l^{-1} H_2S and 100 mM NaCl for 7 days. The initial plant fresh weight was 1.31 ± 0.17 g. Data on biomass production (g fresh weight) and shoot to root ratio (on a fresh weight basis) represent the mean of two experiments with 14 measurements with three plants in each (±SD). Data on dry matter content (DMC; % of fresh weight), chlorophyll content (mg g^{-1} fresh weight) and water-soluble non-protein thiol content (μmol g^{-1} fresh weight) represent the mean of two experiments with 6, 3 and 3 measurements with three plants in each (± SD), respectively. Data on chlorophyll *a* fluorescence (F_v/F_m ratio) represent the mean of two experiments with 12 measurements in each (±SD). Data on the total sulfur and sulfate content (μmol g^{-1} dry weight) represent the mean of 3 measurements with three plants in each (±SD). Different letters indicate significant differences between treatments ($P \leq 0.01$, Student's t-test)

A 7-day exposure of maize to 100 mM NaCl hardly affected plant biomass production and the shoot to root ratio, but resulted in a substantial increase in the dry matter content of both shoot and root, even though aerenchyma had developed in the root (Table 1). Similar to observations with other plant species (Grattan and Grieve 1999; Reich et al. 2016a) exposure to NaCl salinity strongly affected the mineral composition of both shoot and root of maize (Table 3). It resulted in a 70-fold and 28-fold increase in the sodium content in the shoot and root, respectively, accompanied by a strong decrease in the content of calcium, potassium and magnesium in both shoot and root (Table 3). Moreover, salinity exposure resulted in a decrease in the total sulfur content, which could for a greater part be ascribed to a decrease in sulfate content (Tables 1 and 3). Exposure to NaCl salinity resulted in an increase in the chlorophyll content of the shoot, whereas the chlorophyll a/b ratio, chlorophyll *a* fluorescence and the content of the water-soluble non-protein thiols in both shoot and root were unaffected (Tables 1 and 3). Apparently, salinity exposure did not affect the composition and functioning of the photosystems, *viz.* photosynthetic electron transport.

Table 2 The impact of atmospheric H_2S and NaCl salinity on biomass production, chlorophyll content, chlorophyll *a* fluorescence and sulfur metabolite content of maize at anoxic conditions

	Anoxic conditions			
	Control	H_2S	NaCl	H_2S + NaCl
Plant				
Biomass production	5.15 ± 0.72b	7.71 ± 1.12c	2.67 ± 0.47a	2.74 ± 0.33a
Shoot/root ratio	1.42 ± 0.17b	1.77 ± 0.18c	1.23 ± 0.12a	1.23 ± 0.14a
Shoot				
DMC	9.3 ± 0.3a	9.0 ± 0.4a	11.3 ± 0.4b	11.6 ± 0.6b
Chl a + b	0.68 ± 0.10a	0.89 ± 0.08a	0.95 ± 0.10a	1.00 ± 0.03b
Chl a/b	3.1 ± 0.1b	3.0 ± 0.0b	2.7 ± 0.1a	2.8 ± 0.1a
F_v/F_m	0.72 ± 0.07a	0.76 ± 0.09a	0.77 ± 0.04a	0.78 ± 0.06a
Thiols	0.42 ± 0.02a	0.54 ± 0.04b	0.35 ± 0.08a	0.99 ± 0.02c
Sulfate	20 ± 2a	27 ± 5a	36 ± 2b	32 ± 4b
Total sulfur	99 ± 3a	105 ± 3a	108 ± 5a	110 ± 5a
Root				
DMC	6.9 ± 0.5a	6.7 ± 0.5a	7.9 ± 0.6b	8.1 ± 0.7b
Thiols	0.34 ± 0.03a	0.62 ± 0.07b	0.30 ± 0.02a	0.81 ± 0.02c
Sulfate	51 ± 5a	67 ± 12ab	68 ± 3b	57 ± 3a
Total sulfur	111 ± 9a	135 ± 5b	134 ± 5b	127 ± 3ab
Aerenchyma	Present	Present	Present	Present

Plants were exposed to 0.25 μl l^{-1} H_2S and 100 mM NaCl at anoxic root conditions for 7 days. The initial plant fresh weight was 1.23 ± 0.16 g. Data on biomass production (g fresh weight) and shoot to root ratio (on a fresh weight basis) represent the mean of 14 measurements with three plants in each (±SD). Data on dry matter content (DMC; % of fresh weight), chlorophyll content (mg g^{-1} fresh weight) and water-soluble non-protein thiol content (μmol g^{-1} fresh weight) represent the mean of 6, 3 and 3 measurements with three plants in each (±SD), respectively. Data on chlorophyll *a* fluorescence (F_v/F_m ratio) represent the mean of 12 measurements (±SD). Data on the total sulfur and sulfate content (μmol g^{-1} dry weight) represent the mean of 3 measurements with three plants in each (±SD). Different letters indicate significant differences between treatments ($P \leq 0.01$, Student's t-test)

The observed increased plant biomass production upon H_2S exposure did not occur upon a simultaneous exposure to NaCl salinity. Biomass production of these plants was even lower than that of unexposed (control) plants (Table 1). Upon a simultaneous exposure to atmospheric H_2S and NaCl salinity, the chlorophyll content of the shoot, the mineral composition of both shoot and root and the sulfate and total sulfur content of both shoot and root were all quite similar to those observed in the absence of H_2S (Tables 1 and 3). Moreover, the development of aerenchyma in the root was noticeable. Upon a simultaneous exposure to H_2S and NaCl salinity there was not only a substantial increase in the water-soluble non--protein thiol content in the shoot, but also a slight increase in the root (Table 1).

A 7-day exposure of maize to anoxia only slightly affected plant biomass production as compared to oxic conditions and it resulted in an increase in the shoot to root ratio (Tables 1 and 2). Under anoxic conditions, the impact of

Table 3 The impact of atmospheric H_2S and NaCl salinity on the mineral nutrient content of maize

	Control	H_2S	NaCl	H_2S + NaCl
Shoot				
Calcium	198 ± 28b	180 ± 8b	45 ± 4a	46 ± 1a
Copper	0.29 ± 0.03a	0.25 ± 0.03a	0.27 ± 0.01a	0.25 ± 0.02a
Iron	0.74 ± 0.18a	0.96 ± 0.34a	0.87 ± 0.02a	0.86 ± 0.05a
Magnesium	165 ± 19b	152 ± 10b	99 ± 7a	97 ± 1a
Manganese	1.00 ± 0.19a	0.83 ± 0.18a	0.69 ± 0.02a	0.72 ± 0.08a
Molybdenum	0.015 ± 0.001a	0.017 ± 0.003ab	0.026 ± 0.002c	0.020 ± 0.002b
Phosphorus	303 ± 46a	357 ± 37a	309 ± 4a	282 ± 14a
Potassium	1585 ± 38b	1710 ± 26c	1129 ± 157a	907 ± 31a
Sodium	13 ± 3a	19 ± 10a	907 ± 202b	1114 ± 28b
Sulfur	148 ± 20ab	154 ± 7b	123 ± 14a	146 ± 10a
Zinc	0.89 ± 0.13a	0.88 ± 0.24a	0.85 ± 0.13a	0.71 ± 0.07a
Root				
Calcium	257 ± 13b	270 ± 25b	173 ± 11a	140 ± 14a
Copper	0.95 ± 0.16ab	0.67 ± 0.08a	1.02 ± 0.06b	0.92 ± 0.06b
Iron	4.63 ± 0.87ab	2.88 ± 0.37a	6.59 ± 0.41c	4.93 ± 0.58b
Magnesium	217 ± 3b	257 ± 14c	137 ± 13a	112 ± 9a
Manganese	4.09 ± 0.73ab	3.25 ± 0.75a	6.05 ± 0.87b	5.71 ± 0.73b
Molybdenum	0.013 ± 0.001a	0.021 ± 0.003b	0.022 ± 0.001b	0.021 ± 0.002b
Phosphorus	217 ± 2b	239 ± 5c	197 ± 15ab	181 ± 7a
Potassium	1078 ± 38b	1139 ± 14c	378 ± 21a	381 ± 6a
Sodium	72 ± 18a	93 ± 30a	2007 ± 79c	1646 ± 61b
Sulfur	162 ± 9a	174 ± 11a	181 ± 8a	162 ± 7a
Zinc	0.92 ± 0.12b	0.86 ± 0.20ab	0.65 ± 0.12ab	0.53 ± 0.03a

Plants were exposed to 0.25 μl l^{-1} atmospheric H_2S and 100 mM NaCl for 7 days. Data on the mineral nutrient content (μmol g^{-1} dry weight) represent the mean of 3 measurements with three plants in each (±SD). Different letters indicate significant differences between treatments ($P \leq 0.01$, Student's t-test)

exposure to atmospheric H_2S, NaCl salinity and their combination on plant biomass production, chlorophyll content, chlorophyll a/b ratio and chlorophyll *a* fluorescence were quite similar to their impact under oxic conditions (Tables 1 and 2). Again, H_2S exposure resulted in an increased plant biomass production (Table 2). However, upon exposure to NaCl salinity and upon a simultaneous exposure to NaCl salinity and H_2S, the plant biomass was reduced and lower than that of unexposed (control) plants (Table 2). Upon exposure to anoxia the development of aerenchyma in the root was observed at all conditions.

Anoxia exposure affected the mineral composition and resulted in a substantial decrease in the total sulfur (Tables 1 and 2), calcium and magnesium content in both shoot and root as compared to oxic conditions (Tables 3 and 4). The decrease in the plant total sulfur content could predominantly be ascribed to a decrease in sulfate

Table 4 The impact of atmospheric H_2S and NaCl salinity on the mineral nutrient content of maize at anoxic conditions

	Anoxic conditions			
	Control	H_2S	NaCl	H_2S + NaCl
Shoot				
Calcium	124 ± 24b	120 ± 2b	47 ± 8a	42 ± 6a
Copper	0.27 ± 0.04a	0.22 ± 0.01a	0.30 ± 0.07a	0.22 ± 0.02a
Iron	0.64 ± 0.04a	0.89 ± 0.06b	0.85 ± 0.19ab	0.93 ± 0.13b
Magnesium	107 ± 11ab	103 ± 3b	101 ± 20ab	87 ± 6a
Manganese	0.77 ± 0.12a	0.62 ± 0.03a	0.87 ± 0.14a	0.62 ± 0.03a
Molybdenum	0.013 ± 0.001a	0.022 ± 0.001c	0.020 ± 0.002bc	0.018 ± 0.001b
Phosphorus	246 ± 24ab	290 ± 14b	217 ± 10ab	208 ± 6a
Potassium	1484 ± 100b	1703 ± 37c	642 ± 76a	705 ± 45a
Sodium	7 ± 1a	7 ± 0a	1097 ± 120b	945 ± 121b
Sulfur	75 ± 5a	90 ± 0b	86 ± 7ab	98 ± 8b
Zinc	0.56 ± 0.14a	0.49 ± 0.06a	0.93 ± 0.26a	0.64 ± 0.12a
Root				
Calcium	131 ± 15a	127 ± 7a	135 ± 39a	102 ± 15a
Copper	1.47 ± 0.17a	1.68 ± 0.23a	1.93 ± 0.52a	1.77 ± 0.25a
Iron	2.25 ± 0.20a	2.51 ± 0.23ab	3.03 ± 0.25bc	3.66 ± 0.37c
Magnesium	135 ± 9b	155 ± 9b	128 ± 22ab	112 ± 5a
Manganese	4.63 ± 0.51a	5.37 ± 0.65a	7.73 ± 1.49a	6.71 ± 1.00a
Molybdenum	0.013 ± 0.002a	0.029 ± 0.005b	0.018 ± 0.005ab	0.018 ± 0.002a
Phosphorus	212 ± 6a	224 ± 13a	198 ± 21a	195 ± 20a
Potassium	1156 ± 67b	1264 ± 25b	411 ± 27a	464 ± 44a
Sodium	49 ± 8a	38 ± 1a	1437 ± 108b	1463 ± 103b
Sulfur	101 ± 6a	132 ± 9b	118 ± 12ab	129 ± 7b
Zinc	0.78 ± 0.13ab	0.82 ± 0.09b	0.63 ± 0.11ab	0.54 ± 0.04a

Plants were exposed to 0.25 μl l^{-1} H_2S and 100 mM NaCl at anoxic root conditions for 7 days. Data on the mineral nutrient content (μmol g^{-1} dry weight) represent the mean of 3 measurements with three plants in each (±SD). Different letters indicate significant differences between treatments ($P \leq 0.01$, Student's t-test)

content (Tables 1 and 2). Again, H_2S exposure hardly further affected the mineral composition of plants under anoxic conditions (Table 4). Similar to observations under oxic conditions, NaCl salinity strongly affected the mineral composition of both shoot and root of maize (Table 4). It resulted in an increase in the sodium content in both shoot and root, accompanied by a strong decrease in the content of calcium in the shoot and potassium in both shoot and root (Table 4). In contrast to oxic conditions, H_2S exposure resulted not only in an increase in the content of water-soluble non-protein thiols in the shoot, but also in the root (Table 2). Again, exposure to NaCl salinity hardly affected the water-soluble non-protein thiol content in both shoot and root (Table 2). However, a simultaneous exposure of maize to H_2S and NaCl salinity resulted in a more strongly enhanced water-soluble non-protein thiol content in both shoot and root than that observed in the absence of NaCl salinity (Table 2).

From the current study it was evident that maize seedlings were rather unsusceptible to the potentially toxic effects of exposure to H_2S, NaCl salinity and anoxia. Only a combination of NaCl salinity with H_2S and/or anoxia negatively affected plant biomass production. This may indicate that under these conditions, the combination of abiotic stress factors negatively affected the balance between carbon use for structural growth and carbon use for the maintenance respiration required to alleviate the negative effects of the stressors. Furthermore, it was evident that not only anoxia but also NaCl salinity induced the formation of aerenchyma in the roots of maize. It was previously observed that several abiotic stress factors might induce aerenchyma formation in roots (Bouranis et al. 2003; Evans 2003). The enhanced water-soluble non-protein thiol content in the root of maize upon the simultaneous exposure to H_2S, salinity and/or anoxia might be the result of the direct diffusion of atmospheric H_2S via the stomata through the aerenchyma and subsequent metabolism in the root.

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