

# Cell wall invertase-deficient *miniature1* kernels have altered phytohormone levels

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## Abstract

The *Zea mays* (maize) *miniature1* (*Mn1*) locus encodes the cell wall invertase INCW2, which is localized predominantly in the basal endosperm transfer layer (BETL) of developing kernels and catalyzes conversion of sucrose into glucose and fructose. Mutations in *Mn1* result in numerous changes that include a small kernel phenotype resulting from both decreased cell size and number. To explore the pleiotropic effects of this mutation, we investigated the levels of indole-3-acetic acid (IAA), abscisic acid (ABA), salicylic acid (SA), and jasmonic acid (JA) in basal regions, upper regions, and embryos of developing kernels in the inbred line W22. We measured phytohormones from 6 to 28 days after pollination (DAP) in wild type (WT) and two alleles of *mn1*, *mn1-1* and *mn1-89*. IAA was the predominant hormone in kernels, with WT levels of free IAA accumulating over time to more than 2 µg/g of fresh weight. Kernels of *mn1-1* accumulated up to 10-fold less IAA than WT, and levels of IAA sugar conjugates were similarly reduced. Although less abundant, differences were also observed in levels of ABA, JA, and SA between WT and the *mn1* alleles. SA levels were increased by as much as 10-fold in *mn1-1*, and *mn1-89* displayed intermediate SA levels at most timepoints. These findings indicate that invertase-mediated sucrose cleavage directly or indirectly regulates the levels of key plant hormones during seed development.

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## 1. Introduction

Establishment of sink strength in plants is dependent on several factors, including cell number, cell size, and metabolic activity in the sink tissue (Herbers and Sonnewald, 1998). Several lines of evidence indicate that cell wall invertases (INCWs) play a critical role in establishing sink strength (Lara et al., 2004; Roitsch and Gonzalez, 2004;

Weber et al., 1997; Zhang et al., 1996). INCW catalyzes the unidirectional conversion of sucrose into glucose and fructose, which are then taken up by hexose transporters into the sink tissue (Roitsch et al., 2003). The function of cell wall invertase is critical to photoassimilate import into developing seed in maize (Miller and Chourey, 1992; Shannon, 1972), barley (Weschke et al., 2003), bean (Weber et al., 1997), and tomato (Fridman et al., 2004; Godt and Roitsch, 1997).

INCW2 is the primary invertase in immature maize kernels and begins to accumulate at 8 days after pollination (DAP), reaches maximal levels around 12 DAP, then declines steadily as the kernel matures (Cheng et al., 1996). INCW2 is encoded by the *Mn1* locus, and *miniature1* (*mn1*) mutants result from point mutations in this locus that destabilize the *INCW2* transcript or protein (Cheng et al., 1996; Miller and Chourey, 1992), resulting

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in a small kernel phenotype. Multiple alleles of *mn1* have been identified that differ in phenotypic severity. The *mn1-1* allele is caused by a spontaneous mutation that results in a complete loss of *INCW2* expression as detected by northern blot (Talierecio et al., 1999). The *mn1-1* seed mutant shows a loss of 98% of total invertase activity in 12 DAP kernels and a reduction of nearly 70% in mature seed weight. The semi-dominant *mn1-89* allele results from an EMS-induced base change that causes a proline to leucine substitution and likely destabilizes the *INCW2* protein (Carlson et al., 2000). Kernels of *mn1-89* display a loss of 94% of total invertase activity and a 33% reduction in seed weight (Carlson et al., 2000; Cheng et al., 1996). The residual low invertase activity in *mn1-1* kernels is due to a second paralogous gene, *INCW1* (Chourey et al., 2006). It has been hypothesized that the inability of *mn1* to cleave incoming sucrose results in a deficiency in free apoplastic hexose that is predicted to result in decreased sugar uptake and therefore decreased osmotic potential in the developing kernel, thus leading to the decreased seed size phenotype of *mn1-1* (Cheng et al., 1996).

In addition to an overall decrease in seed size, *mn1-1* has decreased numbers of cells and decreased cell size in developing endosperm (Vilhar et al., 2002). Such defects in both cell division and cell enlargement are suggestive not only of altered sugar levels but also of defects in phytohormone pathways. Sugars are known to modulate many critical processes in plant development that are also controlled by phytohormones (Gibson, 2003; León and Sheen, 2003), and in addition to being a major source of carbon, sugars also act as signaling molecules through the action of hexokinase and other undefined sugar sensors (León and Sheen, 2003; Rolland et al., 2002; Rolland and Sheen, 2005; Sheen et al., 1999). Studies have indicated links between glucose levels and cell division and growth (Rolland et al., 2002), processes also controlled by auxin and cytokinin (Coenen and Lomax, 1997; Kende and Zeevaart, 1997). In addition, sugar signaling mutants in *A. thaliana* have been utilized to unravel a complex signaling network that links sugar responses to abscisic acid (ABA) and ethylene (Kim et al., 2004; Laby et al., 2000; Yanagisawa et al., 2003; Zhou et al., 1998). Furthermore, some defective kernel (*dek*) mutants have altered levels of auxin (Lur and Setter, 1993b), indicating the importance of this phytohormone in kernel growth. Profiles from developing maize kernels show that the auxin indole-3-acetic acid (IAA) is abundant in kernels and peaks initially around 12 DAP, a timepoint that coincides with rapid cell division and peak *INCW2* activity. A second IAA peak is observed at 20 DAP, corresponding to the time of rapid embryo growth and IAA conjugate accumulation (Jensen and Bandurski, 1994; Lur and Setter, 1993b). Given the accumulating evidence for linkages between sugar metabolism and phytohormone signals, we hypothesized the *mn1* mutants could have defects in phytohormone levels. We therefore measured levels of carboxylic acid-containing phytohormones IAA, ABA, jasmonic acid (JA) and salicylic acid

(SA) in developing kernels from 6 to 28 DAP and in embryos from 12 to 28 DAP. We found significant decreases in both free and conjugated IAA levels and increases in SA levels in both *mn1-1* and *mn1-89* as compared to wild type, indicating that the loss of cell wall invertase activity in these mutant alleles results in downstream changes in phytohormone levels.

## 2. Results and discussion

The phytohormone quantification method utilized gas chromatography–mass spectroscopy (GC–MS) to measure combined methyl-ester and free-acid forms of several carboxylic acid-containing phytohormones (Schmelz et al., 2003, 2004). The free-acid forms of phytohormones are generally considered to be the active forms *in planta*, whereas the functions of the methyl-ester forms, which naturally exist at much lower levels, are less well understood (Qin et al., 2005; Seo et al., 2001; Shulaev et al., 1997). Because this analysis combines native free acid and methyl ester forms of the phytohormone, we will refer to pools collectively.

Of the phytohormones measured in these studies, IAA was found to be the predominant hormone present in developing kernel tissues at all timepoints except 6 DAP (Fig. 1a–c). In agreement with previous reports of IAA content in developing kernels (Lur and Setter, 1993a), we found that IAA levels plateau initially at 12 DAP, and again later between 20 and 28 DAP. In WT endosperm, IAA accumulated during development and reached levels of more than 2 µg/g fr wt in both upper (Fig. 1a and d) and basal (Fig. 1b and d) kernel regions, whereas IAA levels decreased several-fold after 12 DAP in developing embryos (Fig. 1c and d). Both endosperms and embryos undergo periods of rapid cell division during the studied timeframe, which are essentially complete by 12–16 DAP, followed by periods of cell growth and storage accumulation that continue until 40 DAP (Consonni et al., 2005; Sheridan and Clark, 1987). It was thus somewhat surprising to find that while IAA levels increased throughout development in growing endosperm, they decreased over time in growing embryos. This observation may reflect a greater need for free IAA in expanding endosperm cells, or may simply result from the synthesis and accumulation of vast storage reserves of IAA-sugar conjugates. When compared to WT, *mn1-1* had significantly decreased levels of IAA in both upper and basal kernel regions at 12, 16, 20, and 28 DAP. Similar to the endosperms, we found that embryos from *mn1-1* were reduced in size (data not shown) and also had decreased levels of IAA as compared to wild type at 28 DAP (Fig. 1c). In *mn1-89*, which contains low residual *INCW2* activity and only a moderate reduction in kernel mass, upper and basal kernel regions showed reduced IAA levels intermediate between *mn1-1* and wild type (Fig. 1a and b), and embryo IAA levels similar to those seen in *mn1-1*.

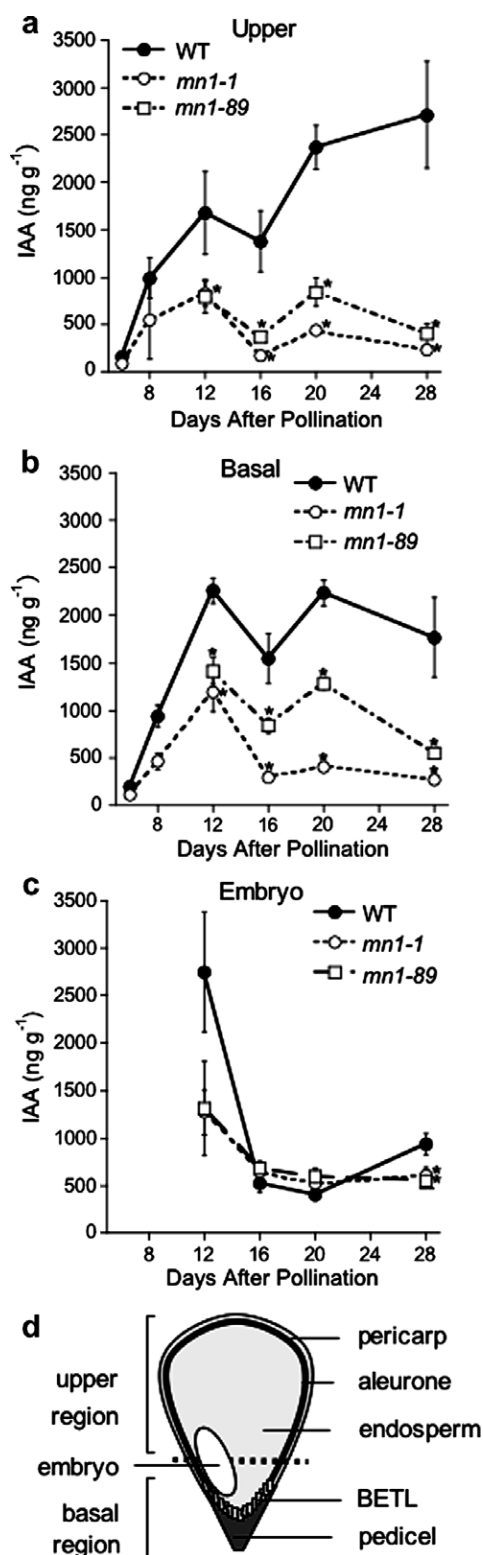


Fig. 1. IAA content in developing kernels. Average ( $n = 3 \pm \text{SEM}$ ) IAA concentration (ng/g fr wt) was measured at 6, 8, 12, 16, 20, and 28 DAP in *mn1-1* (open circles), *mn1-89* (open squares), or wild type (filled circles). Panel a shows upper kernel sections and panel b shows basal kernel sections. Panel c shows IAA levels in embryos from WT and *mn1-1* at 12, 16, 20, and 28 DAP. Panel d shows a diagram of kernel tissues and how the kernel was divided (dashed line) into upper and basal regions and embryos. Asterisks (\*) denote data points that are significantly different from WT ( $P \leq 0.05$ , Student's *t*-test).

Other maize *dek* mutants with aberrant endosperm development have also been found to have altered IAA levels (Lur and Setter, 1993b), indicating that auxin levels and endosperm development are closely linked. The genes defective in these mutants remain to be identified, thus the cause of the *dek* hormone defects is unknown. The determination of IAA deficiencies in the invertase mutants *mn1-1* and *mn1-89* is significant because it suggests connections between sugar metabolism and IAA levels. Our results demonstrate that relative invertase activity and phenotypic severity parallel reductions in IAA levels, suggesting that invertase activity regulates auxin levels, either directly or indirectly, through downstream signals.

To further explore the basis for the decrease in measured IAA levels in *mn1-1*, we measured the levels of total ester-linked IAA conjugates, which represent the major storage form of IAA in maize kernels (Cohen and Bandurski, 1982; Jensen and Bandurski, 1994). Most IAA in developing maize kernels is esterified to various sugars, whereas very little, if any, is found in amide linkages with amino acids or proteins (Cohen and Bandurski, 1982; Jensen and Bandurski, 1994). These IAA-sugar conjugates are believed to function as storage forms of IAA for the developing and germinating embryo (Nowacki and Bandurski, 1980). Multiple ester-linked conjugates including IAA-glucose, IAA-*myo*-inositol, and a large IAA-glycan have been found in maize kernels (Bandurski and Schulze, 1977; Epstein et al., 1980; Jensen and Bandurski, 1994; Nowacki and Bandurski, 1980; Szerszen et al., 1994). These ester linkages can be hydrolyzed relatively easily at room temperature using strong base, thus allowing the measurement of total esterified IAA (Bandurski and Schulze, 1974).

As shown in Fig. 2, we found that sugar conjugates of IAA accumulated to more than 175  $\mu\text{g/g}$  of fr wt, and thus made up the vast majority, about 99%, of the total IAA in developing wild-type kernels. This finding is in close agreement with previous reports measuring the relative levels of

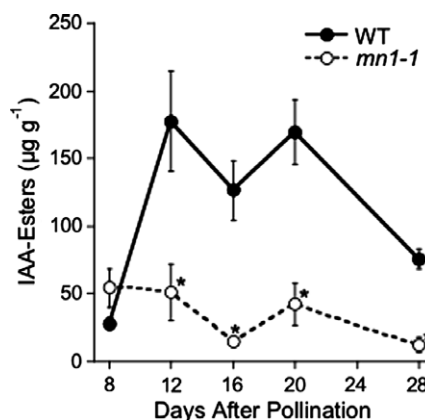


Fig. 2. Total esterified-IAA conjugate content in developing kernels. Average ( $n = 3 \pm \text{SEM}$ ) base-hydrolysable IAA content ( $\mu\text{g/g}$  fr wt) was measured at 8, 12, 16, 20, and 28 DAP in whole kernels of *mn1-1* (open circles), or WT (filled circles). Asterisks (\*) denote data points that are significantly different from WT ( $P \leq 0.05$ , Student's *t*-test).

IAA esters in maize kernels (Cohen and Bandurski, 1982). Similar to levels of IAA, we found that IAA-sugar conjugate levels plateaued at 12 DAP and again at 20 DAP in WT kernels, and were significantly reduced in whole kernels of *mn1-1*. Interestingly in *mn1-1*, there was no further accumulation of IAA-sugar conjugates above the levels present at 8 DAP. This was in sharp contrast to the pattern seen in WT, where IAA-sugar conjugates were at their lowest level at this earliest timepoint. Although future studies will be needed to fully dissect the molecular basis for this decrease in IAA levels in *mn1* mutants, these findings suggest that IAA biosynthetic pathways, rather than conjugate hydrolysis pathways, are most likely affected. If conjugate cleavage was altered in *mn1-1* with no defect in biosynthesis, we would predict either equal or higher conjugate levels in *mn1-1* as compared to WT, rather than the decreased conjugate levels observed. It must also be considered that there could be an increase in oxidative degradation (Beffa et al., 1990) or export (Bangerth, 1989; Else et al., 2004) of IAA as well. Careful measurements of IAA precursors and catabolites, as well as analysis of levels and activities of biosynthetic enzymes will be needed to fully understand the interactions between INCW function and IAA homeostasis.

Additionally, we measured the phytohormones ABA, JA, and SA in developing kernels of WT, *mn1-1*, and *mn1-89* to more fully evaluate the effects of INCW deficiency on phytohormone homeostasis. Levels of JA (Fig. 3) in WT kernel tissues ranged from 5 to 100 ng/g fr wt. JA was increased slightly in *mn1-1* upper kernels (Fig. 3a), but this difference was statistically significant only for the 20 DAP timepoint. JA levels in basal kernel regions of *mn1-1* and upper and basal regions of *mn1-89* were not significantly different from WT (Fig. 3a and b). JA levels in embryos of *mn1-1* and *mn1-89* were significantly higher than WT at multiple timepoints. The slight accumulation of JA in embryos of both mutants and in upper regions of *mn1-1* may reflect sugar signaling events that cause the activity of invertase to lead to reduced JA accumulation, or may represent stress responses caused by metabolic defects. In addition, the JA accumulation could stem from the deficiency in IAA. JA biosynthesis is known to be regulated by a positive feedback mechanism (Turner et al., 2002), and IAA is antagonistic to JA signaling (Baldwin et al., 1997; DeWald et al., 1994). The increase in JA levels could therefore result from the release of inhibition due to decreases in IAA content.

ABA (Fig. 4) in WT kernel tissues ranged from 5 to 150 ng/g fr wt, with the highest levels of ABA in the embryo, slightly lower levels in basal kernel sections, and the lowest levels in upper kernel sections. This is in good agreement with both absolute quantities and relative levels of ABA previously reported for maize (Jones and Brenner, 1987; Tan et al., 1997). Interestingly, levels of ABA were similar between upper and basal regions of WT and *mn1-1*, whereas ABA levels were significantly decreased in *mn1-89* (Fig. 4a and b). In *mn1-89* embryos, ABA was

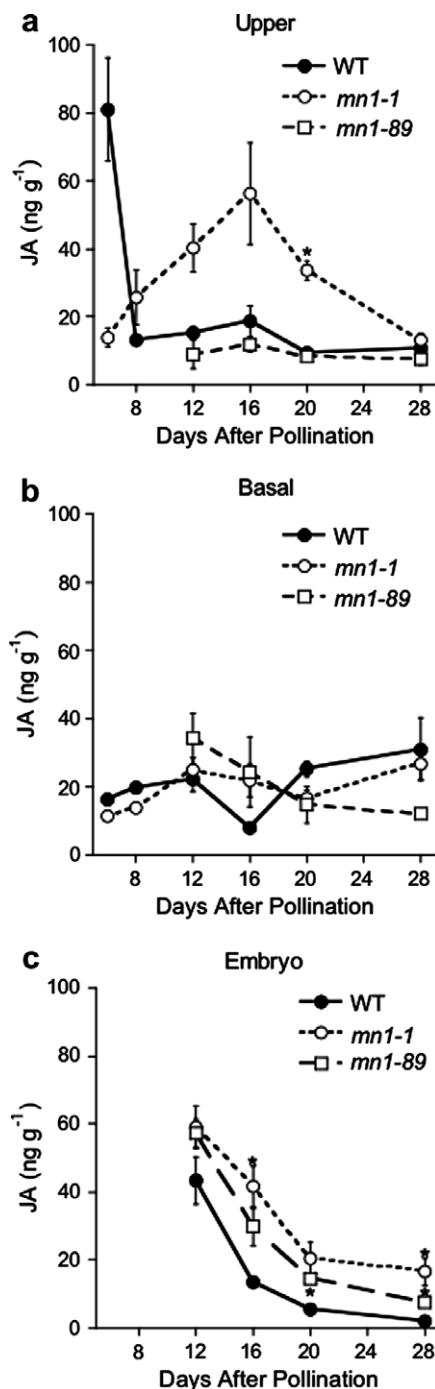


Fig. 3. JA content in developing kernels. Average ( $n = 3 \pm \text{SEM}$ ) JA concentration (ng/g fr wt) was measured at 6, 8, 12, 16, 20, and 28 DAP in *mn1-1* (open circles), *mn1-89* (open squares), or wild type (filled circles). Panel a shows upper kernel sections and panel b shows basal kernel sections. Panel c shows JA levels in embryos from WT and *mn1-1* at 12, 16, 20, and 28 DAP. Asterisks (\*) denote data points that are significantly different from WT ( $P \leq 0.05$ , Student's *t*-test).

significantly increased at 12 DAP, the earliest developmental stage measured (Fig. 4c). It is known that there is extensive crosstalk between ABA and sugar pathways, and many ABA-insensitive mutants are also insensitive to growth inhibition by exogenous sugars (Gibson, 2003;



Kim et al., 2004; Laby et al., 2000). As sugar defects would be expected to be greater in the more severely invertase-deficient *mn1-1* mutant, the finding that ABA levels remain unchanged is somewhat surprising. It is possible, however, that downstream sugar metabolism is differentially affected in *mn1-1* and *mn1-89*. Such a difference in metabolism

could lead to differences in levels of various sugars that may feed into ABA signals.

As shown in Fig. 5, SA was between 10 and 400 ng/g fr wt in WT, and *mn1-1* and *mn1-89* had statistically significant increases in SA at several timepoints in both upper (Fig. 5a) and basal (Fig. 5b) kernel regions. Similar to the

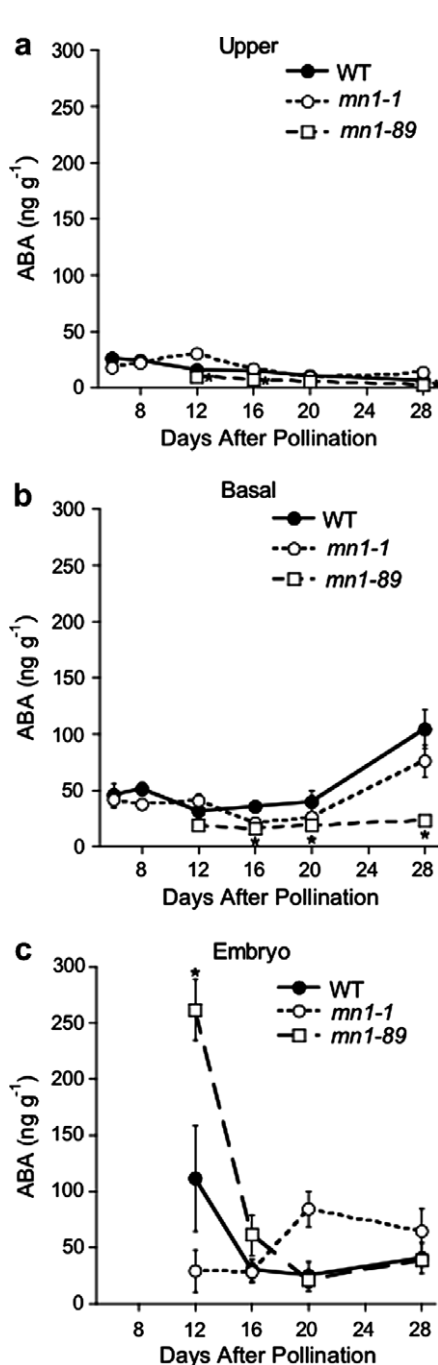


Fig. 4. ABA content in developing kernels. Average ( $n = 3 \pm \text{SEM}$ ) ABA concentration (ng/g fr wt) was measured at 6, 8, 12, 16, 20, and 28 DAP in *mn1-1* (open circles), *mn1-89* (open squares), or wild type (filled circles). Panel a shows upper kernel sections and panel b shows basal kernel sections. Panel c shows ABA levels in embryos from WT and *mn1-1* at 12, 16, 20, and 28 DAP. Asterisks (\*) denote data points that are significantly different from WT ( $P \leq 0.05$ , Student's *t*-test).

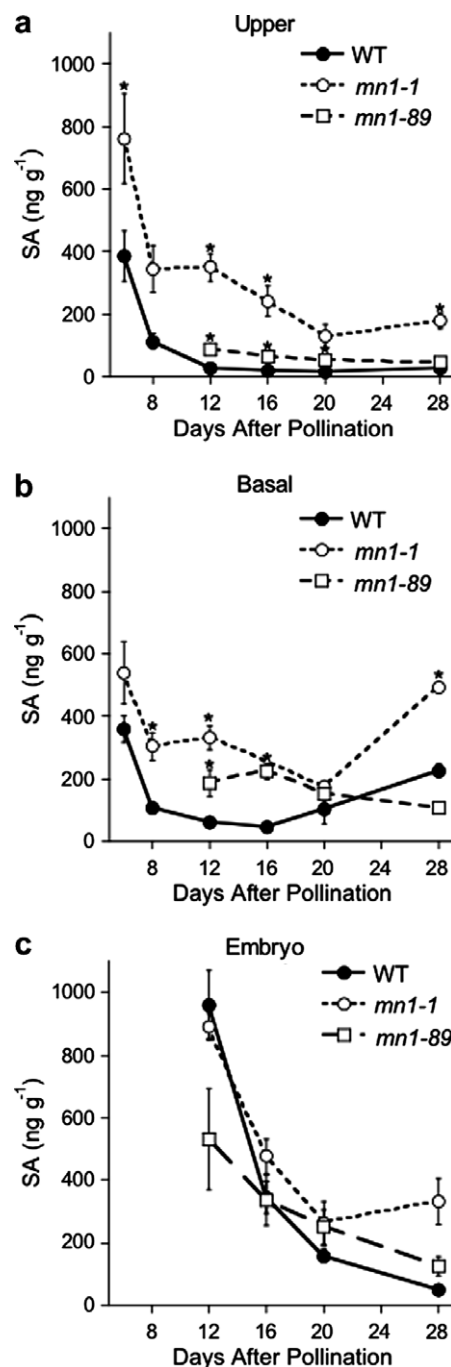


Fig. 5. SA content in developing kernels. Average ( $n = 3 \pm \text{SEM}$ ) SA concentration (ng/g fr wt) was measured at 6, 8, 12, 16, 20, and 28 DAP in *mn1-1* (open circles), *mn1-89* (open squares), or wild type (filled circles). Panel a shows upper kernel sections and panel b shows basal kernel sections. Panel c shows SA levels in embryos from WT and *mn1-1* at 12, 16, 20, and 28 DAP. Asterisks (\*) denote data points that are significantly different from WT ( $P \leq 0.05$ , Student's *t*-test).

trend seen for IAA, *mn1-89* SA levels were between those of WT and *mn1-1*. There were no significant differences in SA levels in embryos. The relevance of the increase in endosperm SA levels relative to the *mn1-1* defect in cell division and cell sizing is not yet clear. Whereas SA and JA are most often associated with defense and stress responses, a great deal of antagonism also exists between these and other phytohormone pathways. The finding that SA is increased in *mn1* kernels likely reflects the complex crosstalk that exists between hormone pathways, as well as an overall stress response. In addition, the increase in SA content also points to hormone and sugar crosstalk, and suggests further interactions between auxin and SA pathways. As IAA has been previously shown to inhibit JA (Baldwin et al., 1997; DeWald et al., 1994), it is tempting to speculate that IAA also inhibits SA accumulation in a similar way. This would explain the elevated levels of SA in the IAA-deficient *mn1* kernels. Alternatively, it is possible that SA inhibits IAA accumulation in developing kernels, and that high SA levels cause the decrease in IAA content. This possibility is supported by the timecourse of hormone levels observed, in which SA is more abundant than IAA at 6 DAP (Figs. 1 and 5) and is significantly higher in *mn1-1* at this time. It is also possible that sugar levels or INCW signals could regulate IAA and SA biosynthesis independently and in opposite directions. Future experiments are needed to further explore the connections between SA, sugars, and IAA.

### 3. Conclusions

The findings presented here suggest complex crosstalk between sugar and hormone pathways in developing kernels. These results suggest that sugar metabolism by invertase in sink tissues may influence sink size and strength by regulating phytohormone levels, particularly levels of IAA. Although auxins have been previously reported to regulate invertases (Roitsch et al., 2003), there has been no evidence to suggest the reverse, nor have we found any reports of sugar levels affecting auxin levels in the literature. Other correlations between changes in sugar content and hormone homeostasis have been reported previously. For example, ethylene and ABA crosstalk with sugar pathways as determined by the characterization of several sugar response mutants (Gibson, 2003; Kim et al., 2004; Laby et al., 2000; Rolland et al., 2002; Sheen et al., 1999; Yanagisawa et al., 2003; Zhou et al., 1998). Additionally, in *Nicotiana tabacum* (tobacco) leaves, cytokinin mediates delayed senescence by increasing cell wall invertase activity and glucose levels. In this work we demonstrated parallels between decreased cell wall invertase activity in *mn1* mutants, decreased IAA content, and increased SA content. There is extensive crosstalk between auxin and cytokinin, and it is possible that some of this interaction is mediated through sugar signals originating from invertase function. Future work will examine the

effects of exogenous sugars on levels of these hormones in *in vitro* kernel culture, and effects of endogenous sugars and metabolites will be examined using sugar-accumulation mutants such as *sh2* and *su1* (Laughnan, 1953) and *sh1* (Chourey and Nelson, 1976). Examination of transcript levels of various IAA and SA biosynthetic enzymes in these mutant backgrounds may allow determination of the molecular mechanisms of the phytohormone changes in *mn1-1* and *mn1-89*.

## 4. Experimental

### 4.1. General experimental

GC–MS was performed as previously described (Schmelz et al., 2003, 2004) using an Agilent 6890 GC connected to a 5973 Mass Selective Detector (Palo Alto, CA). Isotopically labeled internal standards  $^2\text{H}_5$ -IAA and  $^2\text{H}_6$ -SA were purchased from CDN Isotopes (Pointe-Claire, Quebec) and  $^2\text{H}_6$ -ABA was purchased from ICON Isotopes (Summit, NJ).

### 4.2. Plant materials

Mutants *mn1-1* and *mn1-89* are from the genetic background represented by the W22 inbred line. All plants were grown in the field in the summer of either 2005 or 2006 and self-pollinated by hand. Ears were harvested at 8, 12, 16, 20, 24 or 28 days after pollination. Kernels were individually removed with a paring knife to include undamaged base (pedicel) of each kernel, which were immediately frozen in liquid nitrogen. Frozen samples were stored at  $-80^\circ\text{C}$  until analysis.

### 4.3. Hormone analysis

For analysis of 6 and 8 DAP upper and lower kernels, embryos were too small to be visible and were therefore not removed. Kernels were divided with a razor blade just above the pedicel. For 12, 16, 20 and 28 DAP kernels, embryos were first carefully removed, and then remaining endosperm was divided into upper and basal sections (Fig. 1d). Embryos were rinsed briefly in sterile distilled water to remove any residual traces of endosperm, then blotted dry on a kimwipe before weighing. Basal kernel sections included pedicel and closely associated endosperm tissues and represent the sucrose turnover region in developing kernels. Upper kernel sections contained upper endosperm tissue, aleurone, and pericarp and represent the storage region of developing kernels. Each sample was a pool of three upper regions, three lower regions, or three embryos, and triplicate samples were analyzed for each timepoint. All experiments were replicated at least three times with similar results each time. Similar results were obtained for samples from both 2005 and 2006 growing seasons.

Hormone extraction, methylation and analysis was performed as described by Schmelz and others (Schmelz et al., 2004). Briefly, 5  $\mu$ L of 20 ng/ $\mu$ L mass-labeled internal standards mixture and 300  $\mu$ L of 2:1 *m*-pron:H<sub>2</sub>O, pH 2.5, was added to pre-weighed samples in 2 mL screw-cap tubes (Fisher Scientific). Samples were pulverized using zirconium beads (Zirmil) in a FastPrep homogenizer (MP Bio-medicals) and extracted into 1 mL of CH<sub>2</sub>Cl<sub>2</sub>. The organic layer was transferred to a glass vial, derivatized with trimethylsilyldiazomethane (2  $\mu$ L), and quenched with 12% AcOH in hexane (2  $\mu$ L). Samples were heated at 70 °C for 5 min and 200 °C for 2 min, and volatile compounds were collected on SuperQ (Alltech Associates, Inc., Deerfield, IL) and eluted into GC vials with 200  $\mu$ L CH<sub>2</sub>Cl<sub>2</sub>. Hormones were measured by selected ion monitoring and quantified based on internal standards.

#### 4.4. IAA-ester measurement

For each data point, three samples of three whole kernels of 8, 12, 16, 20 or 28 DAP WT or *mml-1* were ground in liquid N<sub>2</sub> and ~100 mg was placed in a 2 mL tube and weighed. 1 N NaOH (100  $\mu$ L) was added, and reactions were incubated at 65 °C for 2 h to cleave ester bonds (Bandurski and Schulze, 1974). Following base hydrolysis, 3 N HCl (50  $\mu$ L) was added to each sample to lower pH to 2.5, and PrOH (200  $\mu$ L) and deuterated IAA (1  $\mu$ g) internal standard (CDN Isotopes, Quebec, Canada) were added. Samples were extracted into CH<sub>2</sub>Cl<sub>2</sub> (1 mL) and an aliquot (100  $\mu$ L) was derivatized and analyzed as described above for hormone analysis.

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