Posttranslational Elevation of Cell Wall Invertase Activity by Silencing Its Inhibitor in Tomato Delays Leaf Senescence and Increases Seed Weight and Fruit Hexose Level

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INTRODUCTION

Invertase (EC 3.2.1.26) hydrolyzes sucrose into glucose and fructose and plays a major role in plant development and in response to biotic and abiotic stresses (Sturm, 1999; Essmann et al., 2008). The resultant hexoses are both important signaling molecules for regulating gene expression and essential substrates for energy (ATP) generation and various metabolic and biosynthetic processes, including starch and cellulose synthesis (Koch, 2004; Rolland et al., 2006). Consequently, invertase activity needs to be tightly regulated in vivo to ensure ordered plant development (Rausch and Greiner, 2004; Ruan and Chourey, 2006). Based on their subcellular locations, invertases are categorized into vacuolar, apoplastic, and cytoplasmic subgroups (Sturm, 1999). Vacuolar invertase has an optimal pH of ~4.5 and may play a role in hexose accumulation and cell expansion in a range of sinks, including sugar beet root (Beta vulgaris; Leigh et al., 1979), maize (Zea mays) pulvinal cells (Long et al., 2002), and tomato fruit (Solanum lycopersicum; Yelle et al., 1991). Decreases in vacuolar invertase activity are associated with responses to low oxygen (Zeng et al., 1999) and drought-induced early seed abortion (Andersen et al., 2002). Apoplastic invertase, with an optimal pH of 4.5 to 5.5, may play diverse roles in phloem unloading (Dickinson et al., 1991; Roitsch et al., 2003), cell division (Weber et al., 1996), and in responses to biotic and abiotic stresses (e.g., Sturm and Chrispeels, 1990; Stitt et al., 1991; McLaughlin and Boyer, 2004; Essmann et al., 2008). Unlike vacuolar or apoplastic invertase, cytoplasmic invertases are not glycosylated and have an optimal pH of 7.0 to 7.8; hence, they also are called neutral/alkaline invertases (Masuda et al., 1987; Sturm, 1999). Both vacuolar and cytoplasmic invertases have an acidic pH value and are soluble, whereas apoplastic invertase has a basic pH value and binds to the cell wall and hence is insoluble.

Over the last two decades, mutational and transgenic approaches have led to significant progress in understanding the role of invertases in plants. Mutation of an apoplastic invertase, INCW2, in maize leads to a miniature seed phenotype (Miller and Chourey, 1992; Cheng et al., 1996). Similarly, antisense suppression of apoplastic invertase in tobacco (Nicotiana tabacum) results in inviable pollen (Roitsch et al., 2003), whereas...
suppression of cell wall and vacuolar invertase in carrot (*Daucus carota*) reduced leaf and taproot growth (Tang et al., 1999). These studies demonstrate the critical roles of invertase in plant development.

Research on invertase regulation has been focused primarily at the transcriptional level (Weber et al., 1996; Lara et al., 2004; Essmann et al., 2008). However, emerging evidence indicates that invertase activity may be subject to posttranslational suppression by its inhibitory protein (Hothorn et al., 2004; Rausch and Greiner, 2004). After biochemical characterization of this inhibitor in the 1960s (Schwimmer et al., 1961; Pressey, 1966), the first cDNA encoding a cell wall invertase inhibitor was cloned three decades later (Greiner et al., 1998). Since then, several cDNAs encoding putative invertase inhibitors have been isolated from various plant species (e.g., Bate et al., 2004; Reca et al., 2008), and Greiner, 2004). After biochemical characterization of this inhibitor in the 1960s (Schwimmer et al., 1961; Pressey, 1966), the first cDNA encoding a cell wall invertase inhibitor was cloned three decades later (Greiner et al., 1998). Since then, several cDNAs encoding putative invertase inhibitors have been isolated from various plant species (e.g., Bate et al., 2004; Reca et al., 2008). Like their target counterparts, these small inhibitory proteins, with molecular masses (M.) ranging from 15 to 23 kD, may be localized to either the cell wall or vacuole (Krauszgrill et al., 1998; Greiner et al., 1998, 2000). Functionality of the inhibitors has been determined largely by in vitro assays of their recombinant proteins (e.g., Greiner et al., 1998; Bate et al., 2004).

Despite these advances, however, little is known regarding the in vivo role(s) of invertase inhibitors (McLaughlin and Boyer, 2004). To our knowledge, there have been no reports thus far on either colocalization of the inhibitor and its target invertase in situ or phenotype from altered expression of endogenous invertase inhibitors in their native plants. Since apoplastic and vacuolar invertases are intrinsically very stable enzymes due to their glycan decoration, control of their activity may be highly dependent on posttranslational mechanisms (Greiner et al., 2000; Rausch and Greiner, 2004). Thus, elucidating the in vivo role of invertase inhibitor should provide new insights into the regulation of invertase and its pivotal role in plant growth and development.

This study explores the role of invertase inhibitor in planta. To achieve this, we cloned a putative invertase inhibitor cDNA, *INVINH1*, from tomato. Its subcellular location and in vivo function were examined by imaging analyses of its protein fused with green fluorescent protein (GFP) combined with ectopic overexpression of *INVINH1* in *Arabidopsis thaliana* and silencing of its expression in tomato. Furthermore, coexpression of *INVINH1* and its target invertase gene was shown in situ, and their physical interaction was demonstrated through coimmunoprecipitation. The data obtained show that *INVINH1* (1) encodes a protein that specifically inhibits the activity of apoplastic invertase in vivo and (2) regulates leaf senescence and seed and fruit development in tomato by capping cell wall invertase activity.

**RESULTS**

Cloning of *INVINH1*, a cDNA Encoding a Putative Invertase Inhibitor That Targets to the Cell Wall

As the first step toward elucidating the role of cell wall invertase inhibitor in tomato, we BLAST-searched various databases and found one putative tomato invertase inhibitor gene sequence (GenBank accession number AJ010943). Full-length cDNA was then cloned from tomato leaves and named *INVINH1*. Sequence analysis revealed an open reading frame of 516 nucleotides for *INVINH1* that encodes 171 amino acid residues (Figure 1A). *INVINH1* has a putative signal peptide of 19 amino acid residues at the N terminus, which results in a predicted M, of 16.6 kD for the mature protein. *INVINH1* differs from SolyCIF, a recently cloned putative invertase inhibitor from tomato with unknown in vivo function (Reca et al., 2008), at amino acid positions 103 and 110 (Pro and Leu in *INVINH1* and Thr and Ile for SolyCIF). BLAST searches revealed *INVINH1* shared high homology exclusively with invertase inhibitors in the first 10 matches. Alignment of *INVINH1* with invertase inhibitors from other species showed the conserved four Cys residues (Figure 1A), a hallmark of all known plant invertase inhibitors (Rausch and Greiner, 2004). Phylogenetically, *INVINH1* was clustered most closely with tobacco apoplastic invertase inhibitor, but distantly with those from maize, rice (*Oryza sativa*), and *Arabidopsis* (see Supplemental Figure 1 online).

*INVINH1* was expressed in both vegetative and reproductive tissues (Figure 1B). Notably, its mRNA level increased as leaves progressed from sink to source stages and as fruit developed from the time of flowering to 20 d afterwards (Figure 1B).

The intracellular localization of *INVINH1* was first deduced using three prediction programs. The bioinformatics analyses unanimously suggested apoplastic targeting of *INVINH1* (Table 1). To verify the predicted apoplastic targeting of *INVINH1*, a construct coding for a *INVINH1*:GFP fusion protein was generated under the control of the cauliflower mosaic virus 35S promoter for stable transformation into *Arabidopsis*. Control plants transformed with 35S:GFP alone displayed fluorescence in nuclei and cytoplasm of root cells (Figures 2A and 2B). By contrast, fluorescence was restricted to cell walls of plants transformed with *INVINH1*:GFP fusion construct (Figures 2C and 2D). Transient expression of the fusion protein in onion epidermal cells consistently revealed fluorescence in cell walls (Figures 2G and 2H), while expression of GFP alone construct showed fluorescent signals in cytoplasm and nuclei (Figures 2E and 2F).

**Overexpression of *INVINH1* Specifically Inhibits Cell Wall Invertase Activity**

To examine whether *INVINH1* functions as a cell wall invertase inhibitor in vivo, the above-described transgenic *Arabidopsis* plants expressing *INVINH1* were assayed for invertase activity in roots where *Arabidopsis* (At) cell wall invertases, At cwINV1 and 4, are expressed (Sherson et al., 2003). Expression of the *INVINH1* in *Arabidopsis* roots was confirmed by RT-PCR analyses using gene-specific primers for *INVINH1*. The 35S:GFP transformed plants were used as a control (Figure 3A). Enzyme assay revealed that, in comparison with control plants, apoplastic invertase activity was reduced by 35 to 55% in roots of three homozygous lines (Figure 3B). Importantly, activities of vacuolar and cytoplasmic invertase were not affected by expression of *INVINH1* (Figure 3C), indicating that *INVINH1* specifically inhibits the activity of apoplastic invertase. The *INVINH1*-expressing *Arabidopsis* plants appeared normal except for an approximate 20% reduction in mature seed weight and earlier appearance of leaf senescence by ~3 d.
Figure 1. Alignment Analysis of INVINH1 and Its mRNA Abundance in Tomato.
To test if overexpression of \textit{INVINH1} in tomato has a similar inhibitory effect on apoplastic invertase activity as observed in \textit{Arabidopsis} (Figure 3B), a 35S:\textit{INVINH1} overexpression construct was introduced into tomato through \textit{Agrobacterium tumefaciens}–mediated transformation. Two primary transgenic lines were generated, and the presence of the transgene was confirmed by PCR analyses. The plants appeared normal during their vegetative phase. However, all transgenic seeds aborted 10 d after flowering (DAF). Consequently, no T1 progeny was produced. Nevertheless, in the two T0 lines, the activity of cell wall invertase, not that of cytoplasmic or vacuolar invertase, was significantly reduced compared with the control plants transformed with a 35S:\textit{β-glucuronidase} (GUS) construct (see Supplemental Figure 2 online).

**Silencing INVINH1 Expression in Tomato Increases Apoplastic Invertase Activity and Delays Leaf Senescence**

To examine the physiological role of \textit{INVINH1}, tomato plants were transformed with an RNA interference (RNAi) silencing construct against \textit{INVINH1}. Nine primary transgenic lines (T0) were identified. The T0 plants were allowed to self-pollinate for seed set. Following segregation analyses of the T1 generation for the presence/absence of the transgene by PCR (see Ruan et al., 2003), three T2 homozygous progeny lines were identified from independent transgenic events for detailed analyses in comparison with their null segregants, which resembled that of wild-type plants.

Figure 4A shows the presence of transgene in the three transgenic lines and its absence in the null. RT-PCR analyses revealed that \textit{INVINH1} transcript became hardly detectable in leaves of the transgenic lines but was readily detected in the null (Figure 4B). This indicates strong silencing of \textit{INVINH1} expression in the transgenic plants. Noteworthy is that silencing of \textit{INVINH1} did not appear to affect the mRNA levels of \textit{Lin6} and \textit{Lin8} (Figure 4B), the only two known apoplastic invertase genes expressed in tomato leaves (Fridman and Zamir, 2003). Repression of \textit{INVINH1} led to a 40 to 65% increase in apoplastic invertase activity in mature leaves in comparison to the null (Figure 4C). No difference was found for activities of either cytoplasmic or vacuolar invertases between transgenic and null plants (Figure 4C).

A remarkable phenotype observed in \textit{INVINH1} RNAi tomato plants was a delay in leaf senescence (Figure 5A). By ~30 d after germination, the first and second true leaves located at the bottom nodes of the null plants displayed a clear sign of senescence (Figure 5A). By contrast, leaves at the same nodes from the transgenic lines remained green (Figure 5A) and did not show yellowing until 5 to 7 d later. This delay in senescence was observed at the basal positions throughout the entire life cycle. In the null, as leaves aged progressively from top to basal nodes, levels of \textit{INVINH1} mRNA increased by approximately twofold (Figure 5B) and protein by approximately threefold (Figure 5C) and cell wall invertase activity dropped by ~90% (Figure 5D) compared with that of expanding leaves (~15% of final leaf area) at the top of the plants. Noticeable also is an increase in transcript abundance of two senescence-associated genes, \textit{SENU2} and \textit{SENU3} (Drake et al., 1996), during leaf senescence (Figure 5B). In contrast with \textit{INVINH1}, the protein abundance of cell wall invertase in the old leaves decreased by approximately twofold compared with that in the expanding leaves (Figure 5C).

Silencing \textit{INVINH1} restored the invertase activity in basal leaves to levels exhibited by young leaves (Figure 5D). This finding demonstrates that the decrease of cell wall invertase activity in old leaves of null plants is largely due to the expression of \textit{INVINH1}.

Leaf senescence of null plants also correlated with the decrease in photosynthetic capacity as implied by the drop of the ratio of variable to maximal chlorophyll fluorescence (Fv/Fm; Figure 5E), similar to that reported by Dai et al. (1999). By contrast, the decrease of Fv/Fm, indicative of disorganization of the photosystem II reaction center (Dai et al., 1999), was much slowed in leaves of the transgenic lines (Figure 5E).

Leaf senescence can be promoted or induced by abscisic acid (ABA; Yang et al., 2002; Gharem et al., 2008). Pertinently, \textit{INVINH1} expression was induced by ABA in germinating tomato seedlings (see Supplemental Figure 3 online). These observations prompted us to examine if ABA-induced leaf senescence is dependent on \textit{INVINH1} expression in tomato.

Figure 6 provides representative results from line 1 with similar data from lines 2 and 8 presented in Supplemental Figure 4 online. It shows that application of ABA to expanded leaves of the

| Program    | Apoplast | ER   | Golgi | CHL  | MT  | Other |
|------------|----------|------|------|------|-----|-------|
| PSORT      | 63.00%   | 14.80% | 14.80% | NA   | NA  | 7.40% |
| Target P   | 88.30%   | NA   | NA   | 2.30% | 2.80% | 9.50% |
| Signal P   | 99.90%   | NA   | NA   | NA   | NA  | 0.10% |

The three intracellular targeting prediction programs used were PSORT (http://psort.hgc.jp/), Target P (http://www.cbs.dtu.dk/services/TargetP/), and SIGNAL P (http://www.cbs.dtu.dk/services/SignalP/). CHL, chloroplast; ER, endoplasmic reticulum; Golgi, Golgi body; MT, mitochondria; NA, not applicable.

*The higher the percentage value, the higher the probability of localization in the indicated subcellular compartment.*

Figure 1. (continued).

(A) Alignment of \textit{INVINH1} with amino acid sequences of invertase inhibitors from rice (OS \textit{INVINH1}, 2, 3, and 6), tobacco (NT \textit{CWINV1} and NT \textit{VINVINH}), maize (ZM \textit{CWINV1} and ZM \textit{VINVINH}2 and 3), and \textit{Arabidopsis} (AT \textit{INVINH}). Four conserved Cys residues are boxed. The gray and black vertical shadings represent regions exhibiting medium and high degrees of amino acid identities, respectively.

(B) Quantitative RT-PCR analyses of transcript levels of \textit{INVINH1} in vegetative and reproductive tomato tissues. Each value is the mean ± se of four biological replicates.
null plants induced leaf yellowing, a sign of senescence, which corresponded to a drop of Fv/Fm ratio (Figure 6B). Quantitative PCR analyses revealed that, similar to that observed in tomato seedlings (see Supplemental Figure 3 online), ABA treatment increased transcript levels of \( \text{INVINH1} \) in the null (Figure 6C), leading to a decrease of cell wall invertase activity (Figure 6D).

This ABA-induced leaf aging was, however, blocked in the transgenic plant (Figures 6A and 6B), where \( \text{INVINH1} \) expression was silenced (Figure 6C) and cell wall invertase activity was significantly increased (Figure 6D). It is noteworthy that although application of ABA decreased transcript levels of cell wall invertase in \( \text{INVINH1} \) RNAi plants (Figure 6C), their invertase activity was significantly higher than those in the null with or without ABA treatment (Figure 6D). This indicates that the invertase activity is largely controlled by \( \text{INVINH1} \).

In addition to its induction of \( \text{INVINH1} \) (Figure 6C), ABA application also increased the transcript level of \( \text{SENU2} \).
The increase in cell wall invertase activity by silencing its inhibitor INVINH1 was silenced (Figure 6C). The induction of SENU3, however, was prevented once INVINH1 was silenced (Figure 6C).

High cell wall invertase activity has been shown to be an essential component of the cytokinin-mediated delay in tobacco leaf senescence (Lara et al., 2004). We thus tested the possible involvement of cytokinin in delayed-leaf aging of INVINH1-silenced tomato plants. Measurement of zeatin and isopentenyl adenosine, two biologically active cytokins (Li et al., 2008), revealed a decrease in the cytokinins contents in old leaves as expected (see Supplemental Figures 5A and 5B online). However, no difference was detected in their contents between the transgenic plants and null during leaf aging, and exogenous application of ABA did not appear to affect the endogenous cytokinin levels (see Supplemental Figures 5A and 5B online).

**Silencing INVINH1 Releases Extra Activity of Apoplasmic Invertase in Developing Tomato Fruit That Increases Seed Weight and Fruit Hexose Level**

INVINH1 is expressed not only in leaves and germinating seedlings (see above) but also in developing fruit (Figure 1B), where a cell wall invertase gene, Lin5, is specifically expressed (Godt and Roitsch, 1997; Fridman et al., 2004). Protein gel blot analyses followed by densitometric quantification revealed that the level of INVINH1 in 20-d-old fruit and seed of the null plant increased by 1.5- and 2.3-fold compared with that in 1-d-old fruit and 10-d-old seed, respectively, while cell wall invertase remained relatively constant during this period (Figure 7A). The increase in INVINH1 transcripts (Figure 1B) and protein (Figure 7A) corresponded to a decrease in cell wall invertase activity in 20-d-old fruit and seed (Figure 7B). Interestingly, vacuolar invertase activity rose in these tissues at 20 DAF compared with that in earlier stages (Figure 7B). The coexpression of INVINH1 and cell wall invertase (see above) inspired us to investigate the impact of silencing INVINH1 on cell wall invertase activity in these reproductive organs and their possible phenotype.

In the INVINH1-silenced lines, the INVINH1 transcript (Figure 8A) and protein (Figure 8B), expressed in 10-d-old fruit and seed of the null plants, became undetectable in these organs. Similar to results in leaves for cell wall invertase genes Lin6 and Lin8 (Figure 4), silencing INVINH1 did not affect transcript or protein levels of the apoplasmic invertase gene, Lin5 (Figures 8A and 8B). However, it did lead to a twofold increase in activity of apoplasmic invertase in fruit and seed compared with the null (Figure 8C) without impacting activities of cytoplasmic and vacuolar invertases.

Sugar measurement revealed ~70% increase in glucose and fructose levels in 10-d-old developing fruit from the transgenic plant compared with levels in the null (Figure 8D). By maturity, fruit glucose and fructose levels were ~50 and 20% higher, respectively, in the transgenic plant than the null (Figure 8E). The fruit size of the transgenic plant remained unchanged.

In developing seed, although the glucose and fructose levels were ~20 to 30% and 10 to 15% lower, respectively, in the transgenic plants (Figure 8D), the transgenic seeds showed 6 to 8% increase in dry weight to fresh weight ratio at 20 DAF and maturity (Figure 9A) and ~10 to 15% increase in protein content in mature seed (Figure 9B). By maturity, transgenic seed weight was ~22% higher than that in the null (0.281 ± 0.021 and 0.345 ± 0.023 g per 100 seeds in the null and transgenic lines, respectively) with an evident increase in seed size (Figure 8F). We observed a similar impact on seed and fruit development when tomato plants were transformed with the same INVINH1 RNAi construct but under the control of a fruit-specific 2A11 promoter (see Chengappa et al., 1999).

The increase in cell wall invertase activity by silencing its inhibitor INVINH and the resultant impact on seed and fruit development (see above) indicate that cell wall invertase is sensitive to posttranslational regulation in vivo. If this is the case, posttranslational suppression of cell wall invertase activity may
negatively impact tomato seed and fruit development. To test this hypothesis, a tobacco (Nt) cell wall invertase inhibitor, Nt CWINVH (Greiner et al., 1998), was overexpressed in tomato under the control of the fruit-specific 2A11 promoter. A total of six primary transgenic lines were obtained. Expression of Nt CWINVH reduced cell wall invertase activity by \( \sim 80\% \) in 10-d-old fruit. Transgenic plants grew normally with slightly smaller fruits. Most strikingly, however, seed number per fruit was reduced to an average of \( 5 \pm 2 \), in comparison with \( 25 \pm 5 \) in wild-type or control plants transformed with 2A11:β-glucuronidase construct. For those viable seeds, PCR analyses of germinating T1 seedlings revealed that they were all null segregants. Similarly, overexpression of INVINH1 driven by the 35S promoter also led to infertile seeds in tomato. These results show that repression of cell wall invertase activity by overexpressing its inhibitors is lethal to seed development in tomato.

**INVINH1 Is Coexpressed with Cell Wall Invertase Gene Lin5 in Fruit Phloem Parenchyma with Their Encoded Proteins Interacting in Vivo**

The observations that INVINH1 and cell wall invertase proteins were coexpressed (Figure 7) and that silencing INVINH1 expression significantly increased cell wall invertase activity in fruit and seed (Figure 8) indicate that INVINH1 and the cell wall invertase gene Lin5 could be spatially expressed in the same cell type(s) in young tomato fruit. To examine this possibility, in situ hybridization was performed on sections of 1-d-old fruit with specific RNA probes for INVINH1 and Lin5. In comparison with the sense probe control (Figure 10A), hybridization with the antisense INVINH1 probe revealed weak but detectable signals of mRNA in vascular tissues of pericarp and placenta (Figure 10B). Under high magnification (Figure 10C), the INVINH1 mRNA signal became more evident in the placenta vasculature connecting the seed. Significantly, hybridization with an antisense probe against Lin5 also revealed strong mRNA signals in the placental vascular region extending toward the seed and weak signals in pericarp vasculature (Figure 10E) in comparison with its sense control (Figure 10D). The placental vascular bundles in Figure

![INVINH1 and CWINV](image)

**Figure 5.** Leaf Senescence Was Delayed in the INVINH1-Silenced Tomato Plant.

(A) The first and second true leaves from the bottom of the null plant turned yellow and curly at \( \sim 30 \) d after germination. By contrast, the leaves at these positions of the three transgenic lines remained green at this stage.

(B) Quantitative PCR analyses revealed that the mRNA levels of INVINH1 as well as two senescence-associated genes, SENU2 and 3, increased as the leaf aged at the bottom of the null plant. Top, middle, and bottom leaves represented 5-d-old expanding, 20-d-old expanded, and 30-d-old leaves, respectively.

(C) Protein gel blots for the invertase inhibitor and cell wall invertase in leaf extracts from the null plants probed with antibodies against INVINH1 and maize cell wall invertase, respectively. Note the increase in INVINH1 abundance but a decrease for CWINV as leaves aged.

(D) As leaves developed, cell wall invertase activity in the null plant (white bar) decreased, being highest in the young leaves (\( \sim 15\% \) of final leaf area) on top of the plants and lowest at the bottom leaves. High invertase activity was, however, maintained in the transgenic lines, particularly in the bottom leaves. Each value is mean \( \pm \) SE of three biological replicates. An asterisk indicates a significant difference (t test, *P < 0.05; **P < 0.01).

(E) The progressive decrease of photosynthetic efficiency, Fv/Fm ratio, as leaves aged, observed in the null, was much slower in the INVINH1 silenced lines. Each value is mean \( \pm \) SE of three biological replicates.
10E were cut transversely. For a better comparison with spatial localization of INV1H1 mRNA (Figure 10C), a longitudinal view of placenta connecting the seed is shown in Figure 10F. This displayed strong Lin5 mRNA signals in the vasculature interconnecting the two tissues, resembling that of INV1H1 mRNA (Figures 10F versus 10C).

A close examination of the placenta vascular bundle (Figure 10E) revealed the mRNA of Lin5 was mainly localized in phloem parenchyma cells but not in sieve element and companion cells and xylem elements (Figure 10G). Relatively weak signals of Lin5 also were observed in xylem parenchyma cells (Figure 10G). Identity and position of these cell types were judged by staining a consecutive section with Aniline blue (Figure 10H). This displayed fluorescent signals of callose in sieve element but not in adjacent companion cells. Xylem elements exhibited autofluorescence due to their thickened and lignified cell walls (Figure 10H). Importantly, a similar cell-specific expression pattern was observed for INV1H1 mRNA in the placenta vasculature. The transcript was predominantly detected in phloem parenchyma and weakly in xylem parenchyma (Figure 10I). Colocalization of INV1H1 and Lin5 transcripts also was observed in phloem parenchyma of fruit pericarp.

Coexpression of INV1H1 and Lin5 mRNAs (Figure 10) and proteins (Figures 7 and 8) and the significant elevation of cell wall invertase activity by silencing expression of INV1H1 (Figure 8) strongly indicated that INV1H1 and Lin5 proteins physically interact in vivo. To directly demonstrate this, coinmunoprecipitation experiments were conducted. For these experiments, the entire coding regions of INV1H1 and Lin5 were fused with those of GFP and a hemagglutinin (HA) tag (see Wood et al., 2006), respectively. Constructs harboring fused INV1H1-GFP and Lin5-HA were cotransformed into Arabidopsis. Analyses

Figure 6. Silencing INV1H1 in Tomato Delayed ABA-Induced Leaf Senescence.

(A) ABA induced leaf senescence (yellowing in circle) in the null but not in the INV1H1-silenced plant.

(B) ABA reduced maximum photosynthetic efficiency (Fv/Fm) in the null plant but not in the INV1H1-silenced plant.

(C) ABA treatment enhanced the expression of INV1H1 and SENU2 and 3 in mature leaves from the null. The induction was abolished for INV1H1 and SENU3 and reduced for SENU2 in the RNAi-silenced INV1H1 plants.

(D) Cell wall invertase activity was reduced by ABA treatment in mature leaves from the null. Silencing INV1H1 expression increased cell wall invertase activity above the level in the null, even after treatment with ABA.

Each value in (B) to (D) is the mean ± SE of four biological replicates. An asterisk indicates a significant difference (t test, *P < 0.05; **P < 0.01).
revealed that anti-GFP antibody not only immunoprecipitated INVINH1-GFP (Figure 11A) but also coimmunoprecipitated Lin5-HA (Figure 11B) from the protein extracts. Similarly, anti-HA antibody not only immunoprecipitated Lin5-HA (Figure 11C) but also coimmunoprecipitated INVINH1-GFP (Figure 11D). Plants transformed with INVINH1-GFP or Lin5-HA alone were used as controls, which showed no Lin5-HA and INVINH1-GFP signals when extracts were immunoprecipitated by antibody against GFP and HA, respectively. These results show that INVINH1 and Lin5 interacted in vivo.

The physiological significance of coexpression of INVINH1 and Lin5 in phloem parenchyma (Figure 10) and their interaction (Figure 11) would depend upon, in part, the cellular pathway of phloem unloading of sucrose in tomato fruit and seed (see Ruan and Patrick, 1995). Thus, experiments were conducted to determine if a symplasmic pathway is operative in young tomato fruit and seed. To this end, a phloem-mobile symplastic fluorescent probe, carboxyfluorescein (CF), was ester-loaded into shoots through their cut ends for 24 h (Ruan et al., 2001). Subsequent unloading pattern of CF from the phloem of the 1-d-old fruit through plasmodesmata was monitored in situ. Figure 12A shows that the fluorescent CF signal spread into the placenta but was unable to travel beyond the vascular interface bordering the developing seed. The same image was viewed under bright field to show the seed and location of the vascular bundle (Figure 12B). Interestingly, within the pericarp, CF signals moved readily from the phloem to the surrounding parenchyma storage cells (Figure 12A) as previously reported (Ruan and Patrick, 1995). This observation indicates that the feeding time was sufficient for the CF to be transported extensively in the tomato fruit if a symplasmic continuity exists. Indeed, lack of CF movement from the placenta to the seed (Figure 12A) was observed even if the feeding time was extended to 48 h.

**DISCUSSION**

Posttranslational regulation of invertase activity by its inhibitory proteins is postulated to play an important role in controlling sucrose use and plant development (Rausch and Greiner, 2004; Ruan and Chourey, 2006). However, to our knowledge, there has been no in vivo evidence thus far regarding the presence of such a control and its developmental and physiological significance in their native plants. Here, we provide data showing that INVINH1, cloned from tomato, specifically inhibited cell wall invertase activity in planta. Furthermore, abolishing this inhibition by silencing INVINH1 in tomato elevated cell wall invertase activity, which (1) delayed ABA-induced leaf senescence and (2) increased seed weight and fruit sugar level, probably by enhancing apoplastic sucrose hydrolysis, phloem unloading, and hexose accumulation. These findings provide novel insights into the posttranslational regulation of cell wall invertase activity in relation to plant development and offer new opportunities to improve plant performance through manipulating the interaction of cell wall invertase and its inhibitor.

**INVINH1 Functions as a Cell Wall Invertase Inhibitor in Planta**

Several lines of evidence demonstrate that INVINH1 specifically inhibits cell wall invertase activity in vivo. First, protein targeting analyses using three different programs all predicted an apoplastic localization of INVINH1 (Table 1). Consistent with this prediction, INVINH1 shared the highest sequence homology with a tobacco apoplastic invertase inhibitor (see Supplemental Figure 1 online). Second, imaging analyses of INVINH1-GFP fusion protein confirmed its localization in cell walls (Figure 2). Third, overexpression of INVINH1 in Arabidopsis and tomato reduced the activity of apoplastic invertase (Figure 3; see Supplemental Figure 2 online), whereas silencing its expression in tomato elevated the invertase activity (Figures 4, 6, and 8). These observations demonstrate the functionality of INVINH1 in inhibiting apoplastic invertase activity. Importantly, altered INVINH1 expression did not affect activities of vacuolar or cytoplasmic invertase (Figures 3 and 4), indicating a high specificity of INVINH1 against cell wall invertase. Finally, the colocalization of INVINH1 with the cell wall invertase gene Lin5 in fruit vascular tissues (Figure 10) and the physical interaction of their encoded proteins (Figure 11) provide further evidence that INVINH1 targets cell wall invertase in situ. Collectively, the data demonstrate that INVINH1 functions as a cell wall invertase inhibitor in vivo.

The target of cloned putative invertase inhibitor has been deduced largely from in vitro assay of the recombinant proteins (e.g., Greiner et al., 1998; Bate et al., 2004), which may not
necessarily reflect their roles in vivo (Greiner et al., 2000). For example, while GFP fusion analysis of a putative invertase inhibitor, SolyCIF, indicated its apoplastic localization, in vitro assay showed it inhibited vacuolar not cell wall invertase (Reca et al., 2008). In some cases, functional identity of invertase inhibitors has been studied by expressing the protein in a different plant species. For example, expression of a tobacco invertase inhibitor (Nt VIF) in potato (Solanum tuberosum) reduced vacuolar invertase activity and prevented hexose accumulation in potato tubers (Greiner et al., 1999). This approach, however, does not reveal in vivo functionality of the inhibitors and their potential physiological roles in their native plants (Rausch and Greiner, 2004; Lara et al., 2004).

In view of the above analyses, our results are of particular significance for three reasons. First, INVINH1 colocalization and interaction with cell wall invertase in vivo to specifically inhibit invertase activity represents an example of invertase inhibitor function in its native plant. Second, silencing INVINH1 expression in tomato increased cell wall invertase activity by 40 to 65% in mature leaves (Figures 4 to 6) and by twofold in developing fruit and seed (Figure 8) without a significant impact on mRNA levels of cell wall invertase genes. These data show that a high proportion of cell wall invertase activity is under posttranslational control of INVINH1 in tomato. Third, release of the extra cell wall invertase activity by silencing INVINH1 has profound impacts on the development of both vegetative and reproductive tissues (see below).
Expression of INVINH1 Is Required for ABA-Induced Leaf Senescence

An important observation in INVINH1-silenced tomato plants was a delay in leaf senescence, accompanied with maintenance of leaf cell wall invertase activity and photosynthetic capacity (Figure 5). High cell wall invertase activity is required for cytokinin-mediated delay of leaf senescence in tobacco (Lara et al., 2004), whereas a decrease in cell wall invertase activity is associated with salinity-induced leaf senescence in tomato (Ghanem et al., 2008). However, it remains unknown from these studies if the invertase activity is under regulation of its endogenous inhibitor and if such a control is physiologically relevant to leaf senescence (Lara et al., 2004; Rausch and Greiner, 2004).

The finding from our study that silencing INVINH1 in tomato specifically increased cell wall invertase activity (Figure 4) demonstrates that enzyme activity is limited by its inhibitory protein in vivo. In null or wild-type plants, this limitation is most pronounced in senescencing leaves where mRNA levels of INVINH1 increased and cell wall invertase activity decreased to <10% of that in young leaves (Figure 5). A similar increase in expression of cell wall invertase inhibitor has been seen in senescencing leaves of tobacco (Greiner et al., 1998), indicating that the rise of invertase inhibitor expression may be required for leaf senescence in a variety of plant species. This notion is substantiated by the observation that silencing INVINH1 expression restored cell wall invertase activity in old leaves to the level of young leaves, which delayed leaf aging (Figure 5).

Leaf senescence is a highly regulated process. Among various factors, ABA is known to induce or be involved in leaf senescence in some species (e.g., Yang et al., 2002; Ghanem et al., 2008; also see Figure 6A). The underlying molecular mechanism, however, is unclear. Since ABA induced expression of cell wall invertase inhibitor in leaves of tomato (Figure 6C) and tobacco (Rausch and Greiner, 2004), we hypothesized that expression of INVINH1 may be required for ABA-induced leaf senescence in tomato. Our analyses revealed that the increase in INVINH1 transcript by ABA (Figure 6C) reduced cell wall invertase activity in null or wild-type tomato leaves (Figure 6D). On the other hand, silencing INVINH1 expression restored cell wall invertase activity even after ABA treatment (Figures 6C and 6D), leading to the inability of ABA to induce leaf senescence (Figure 6A). The data show that expression of INVINH1 is a prerequisite for ABA-induced leaf senescence in tomato.

The above analyses concur with previous findings (Lara et al., 2004) that low cell wall invertase activity is required for leaf senescence but provide new insights into this process that (1) the decrease of cell wall invertase activity in old leaves is largely due to the expression of its endogenous inhibitor and (2) the ABA-induced tomato leaf senescence is dependent on the expression of inhibitor gene, INVINH1. Moreover, this induction is independent of cytokinin, since silencing INVINH1 or application of ABA did not affect the endogenous cytokinin levels (see Supplemental Figure 5 online).

Leaf senescence is characterized by remobilization of nutrients, including carbon and nitrogen (Drake et al., 1996; Ghanem et al., 2008). Expression of INVINH1 could decrease hexose levels in leaf apoplasms through inhibition of cell wall invertase activity. Reduction of hexose levels has been shown to stimulate senescence in tomato leaves (Dai et al., 1999) and maize ovaries (McLaughlin and Boyer, 2004). It is also possible that prevention of sucrose hydrolysis in cell walls by INVINH1 could decrease hexose levels in their old leaves. In situ assay of leaf apoplastic and symplasmic sugar levels in the transgenic plants may further clarify the role of INVINH1 in carbon remobilization and ABA-induced leaf senescence.
**INVINH1 expression may also be required for the induction of senescence-associated genes, such as those encoding Cys proteases responsible for nitrogen remobilization (see Chen et al., 2002). In agreement with this is the coexpression of INVINH1 and two senescence-related Cys protease genes, SENU2 and SENU3 (Drake et al., 1996) during leaf aging (Figure 5B) and their coinduction by ABA (Figure 6C). Indeed, silencing INVINH1 reduced the expression of SENU2 and SENU3 in comparison with the null and even rendered SENU3 uninducible by ABA (Figure 6C), indicating INVINH1 may act upstream of this SENU3 induction.**

**INVINH1 Controls Seed and Fruit Development by Capping the Cell Wall Invertase Activity at the Phloem Unloading Sites**

Silencing INVINH1 resulted in not only a delay in leaf senescence (see above) but also a significant increase in fruit hexose levels and seed weight (Figure 8). The latter can be attributed to doubling cell wall invertase activity in developing fruit and seed of INVINH1-silenced plants (Figures 8A to 8C), owing to the specificity of INVINH1 against cell wall invertase (see previous discussion).
The role of cell wall invertase in sink development has been demonstrated in a range of plant species through inhibition of its gene expression (Cheng et al., 1996; Tang et al., 1999; Roitsch et al., 2003; Zanor et al., 2009). However, little is known regarding the potential effects on those sinks once the invertase activity is elevated. In this context, our study represents a remarkable example of positive impacts on fruit and seed development by increases in cell wall invertase activity. Importantly, this is achieved by releasing extra invertase activity through silencing its endogenous inhibitor \textit{INVINH1} without introducing a foreign invertase gene, commonly used in overexpression studies (e.g., Dickinson et al., 1991; Herbers et al., 1996). This posttranslational approach permits assessment of the functional plasticity of endogenous cell wall invertase in planta, which is intractable in ecotopic overexpression studies.

It is of significance to note the cell-specific colocalization of \textit{INVINH1} and \textit{Lin5} mRNAs in young tomato fruit (Figure 10) and the physical interaction of their encoded proteins (Figure 11). Among four cell wall invertase genes in tomato, \textit{Lin5} is the predominant member expressed in developing fruit and seed, whereas the \textit{Lin6} transcript level is only \(-10\%\) of \textit{Lin5}, and \textit{Lin7} and \textit{Lin8} mRNA are undetectable (Godt and Roitsch, 1997; Fridman and Zamir, 2003). In contrast with the invertase gene family, \textit{INVINH1} appears to be the only cell wall invertase inhibitor gene in tomato based on our database searches. \textit{Lin5} is expressed mainly in fruit vascular tissue and genetically linked with fruit sugar levels (Fridman et al., 2004) and seed development (Zanor et al., 2009). However, the cellular sites of \textit{Lin5} expression and its possible control by \textit{INVINH1} have not been resolved in previous studies.

Here, we show that \textit{Lin5} transcript colocalized with that of \textit{INVINH1} in phloem parenchyma cells of tomato fruit (Figures 10G to 10I). Importantly, in vasculatures connecting seeds, their

**Figure 11.** Interaction of \textit{INVINH1} and \textit{Lin5} in Vivo Measured by Coimmunoprecipitation.

(A) Protein extracts were immunoprecipitated with antibody against GFP. The subsequent protein gel blot was probed with anti-GFP antibody. Note the detection of the \textit{INVINH1}:GFP fusion protein from \textit{Arabidopsis} cotransformed with \textit{INVINH1}:GFP and \textit{Lin5}:HA or with \textit{INVINH1}:GFP alone (positive control) but not those with \textit{Lin5}:HA (negative control).

(B) The protein extracts immunoprecipitated with antibody against GFP in (A) were probed with anti-HA antibody. This detected HA-tagged \textit{Lin5} fusion proteins from the plants cotransformed with \textit{INVINH1}:GFP and \textit{Lin5}:HA, confirming that \textit{Lin5} was coimmunoprecipitated with \textit{INVINH1}. As expected, the anti-HA antibody did not detect signals from proteins immunoprecipitated by anti-GFP antibody from plants transformed with \textit{INVINH1}:GFP or with \textit{Lin5}:HA alone.

(C) Protein extracts immunoprecipitated with antibody against HA. The subsequent protein gel blot was also probed with anti-HA antibody. Note the detection of the \textit{Lin5}:HA fusion protein from \textit{Arabidopsis} cotransformed with \textit{INVINH1}:GFP and \textit{Lin5}:HA or with \textit{INVINH1}:HA alone (positive control) but not those with \textit{INVINH1}:GFP (negative control).

(D) Protein extracts immunoprecipitated with anti-HA antibody in (C) were probed with anti-GFP antibody. This detected GFP-tagged \textit{INVINH1} fusion protein from plants cotransformed with \textit{INVINH1}:GFP and \textit{Lin5}:HA, confirming that \textit{INVINH1} was coimmunoprecipitated with \textit{Lin5}. Protein extract from plants transformed with \textit{INVINH1}:GFP or with \textit{Lin5}:HA alone served as negative controls here.

**Figure 12.** A Symplamtic Fluorescent Dye, CF, Failed to Move from the Vascular Region of the Fruit Placenta to the Seed.

(A) A free-hand section of 1-d-old fruit, showing that CF moved to the vascular region of the placenta (p) but did not spread beyond the interface (asterisk) with the seed (s). Also note the extensive CF movement from the vascular bundle (v) of outer pericarp (pe) to the surrounding storage parenchyma cells.

(B) The same section in (A) but viewed under bright field to show the position of the seed and fruit cell types. Bars = 500 \(\mu\)m.
mRNA signals were confined to the placenta and seed coat interface (Figures 10C and 10F). These observations suggest that phloem parenchyma at the border of placenta and seed is the likely cellular site for an apoplastic phloem unloading of sucrose. This notion is supported by the finding that a symplasmic fluorescent dye, CF, spread in placenta but did not reach developing seeds (Figure 12), demonstrating a symplasmic discontinuity at the interface between the two tissues. Therefore, phloem unloading at this region must follow an apoplastic pathway, where the joint action of INVINH1 and Lin5 would determine rates of sucrose hydrolysis in cell walls for delivering hexoses to the developing seeds.

The above analyses suggest that Lin5 plays an important role in extracellular sucrose hydrolysis at the placenta phloem parenchyma cells with its activity capped by the inhibitor, INVINH1. Consistent with this conclusion, silencing INVINH1 indeed released extra invertase activity (Figure 8C). This likely enhances sucrose degradation in the apoplasms in facilitating its phloem unloading down a concentration gradient. Consequently, more hexoses may be available in the placenta apoplasms for their transport to, and use within, the developing seeds. These hexoses may serve as signals to stimulate cell division (Weber et al., 1996) and accumulation of dry matter and proteins (Figure 9), leading to an increase in seed weight and size (Figure 8F) and a decrease in its bulk soluble sugar levels (Figure 8D).

In the fruit pericarp, phloem unloading follows a symplasmic route from 0 to 15 DAF (Figure 12; Ruan and Patrick, 1995). Thus, cell wall invertase in pericarp phloem (Figures 8 and 10) likely hydrolyses sucrose leaked from the unloading cells. At this early stage, tomato fruit undergoes rapid cell division with small intercellular spaces (Ho, 1988). Therefore, it is possible that cell wall invertase activity may generate a high hexose level and hence an osmotic potential in the phloem parenchyma apoplasms (see Essmann et al., 2008), which would lower the turgor of those cells for symplasmic unloading down a turgor gradient (Patrick, 1997). Elevation of cell wall invertase activity by silencing INVINH1 may increase apoplastic hexose levels and hence enhance its osmotic effect to facilitate symplasmic sucrose unloading to the pericarp phloem parenchyma, leading to increased sugar levels in fruit (Figures 8D and 8E).

Alternatively, hexoses released from the enhanced invertase activity (Figure 8C) could serve as signaling molecules (Weber et al., 1996; Koch, 2004; Rolland et al., 2006) that may alter the biochemical or developmental processes of tomato fruit, favoring hexose accumulation. This might be achieved by shifting the cellular carbon flux from metabolism and biosynthesis to hexose accumulation (see Ruan and Patrick, 1995). Support for this possibility comes from the findings that silencing INVINH1 led to a reduction in 10-d-old fruit of both (1) activity of sucrose synthase, a major enzyme involved in starch biosynthesis in tomato fruit (Wang et al., 1993), by ~50% (see Supplemental Figure 6 online), and (2) starch content by ~35% in the transgenic lines. Further studies are underway to elucidate the potential sugar signaling pathways that lead to the shift from sucrose metabolism to hexose accumulation in the INVINH1-silenced tomato fruit.

METHODS

Plant Material

Tomato (Solanum lycopersicum XF-2) were grown in pots in the greenhouse at 25°C with a 16-h photoperiod. The flowers were tagged at anthesis to determine fruit age. For plants grown in vitro, seeds were germinated on MS medium (Murashige and Skoog, 1962) with 16 h of light at 25°C and 8 h of darkness at 22°C.

Cloning of Tomato INVINH1 and Sequencing

Total RNA was isolated from developing tomato leaves using a Plant RNeasy kit from Invitrogen and reverse-transcribed into cDNA. Database searching revealed a putative tomato invertase inhibitor cDNA, INVINH1 (GenBank accession number AJ010943). Full-length INVINH1 was cloned from the cDNA by PCR using the following primers: 5'-ATGAAATTTTGATTTCCTC-3' and 5'-TTACCAATAATTTCTTACAA-3'. The PCR product obtained was fully sequenced and cloned into pGEMT vector (Promega).

Phylogenetic Analysis

Alignment of amino acid sequences and phylogenetic analyses were conducted using MEGA, version 3.0 (Kumar et al., 2004) with the UPGMA method followed with phylogeny test options of bootstrap 1000 trials and seed number of 51,580. Alignments used for phylogenetic analysis are provided in Supplemental Data Set 1 online.

Gene Constructs and Plant Transformation

For constructing the 35S:INVINH1 RNAi vector, a 303-bp INVINH1 fragment was amplified from the INVINH1 cDNA, starting from 76 bp downstream of the start codon. The forward primer sequence incorporated restriction sites for XbaI and BamHI at the 5’ end: 5'-GACTCTTA-GAACAAGTGAAGAGACACCAC-3'. The reverse primer incorporated SacI or SmaI restriction sites at the 5’ end: 5'-GACTTTGATCCCAACACATTCCATATTATGCAA-3'. The introton of AWRY3 was amplified by a primer set with BamHI and SacI restriction sites incorporated at the 5’ and 3’ ends, respectively: forward, 5'-GACTCTTAAGTGAAGAGAG-CAACCCATTT-3', reverse, 5'-GACTTTGATCCCAACACATTCCATATTATGCAA-3'. The amplified fragments were digested with respective enzymes and cloned sequentially into pBlueScript KS+/- (Stratagene) in a sense-intron-antisense orientation. The invert-repeat RNAi gene cassette was then released with SacI and XbaI and cloned into pCAMBIA1300 vector (Cambia) downstream of the 35S promoter.

A fruit-specific RNAi construct against INVINH1 was made by replacing the 35S promoter in the 35S:INVINH1 RNAi construct with a fruit-specific 2A11 promoter (Chengappa et al., 1999).

The overexpression construct for INVINH1 or a tobacco cell wall invertase inhibitor (Nt CIF; Greiner et al., 1998) was made by cloning the respective full-length cDNA into pCambia1300 vector downstream of the 35S promoter.

For constructing 35S:INVINH1:GFP, the full-length INVINH1 cDNA without its stop codon was cloned into pEGFP vector (Clontech) upstream of EGFP. The fragment of INVINH1-GFP was released from the vector by digestion with XbaI and cloned into pCambia1300 vector downstream of the 35S promoter.

Tomato plants were transformed with each of the above silencing or overexpression constructs according to D’Aoust et al. (1999). Briefly, seeds were germinated in half-strength MS medium. Expanded cotyledons of 10-d-old seedlings were excised for infection with an Agrobacterium tumefaciens culture containing the appropriate gene construct. After 2 weeks in hygromycin selection medium, the calli were
excised from the cotyledons and transferred to an organogenesis medium. The shoots grown from the calli were screened for their ability to form roots on hygromycin-containing medium. The incorporation of the transgene was monitored by PCR analyses with the following primer set: 5′-CTCGGATTCCATGGC-3′ and 5′-CAACGATGCAAGACACACACCA-3′. The expected 571-bp PCR product includes 268 bp of 35S promoter and 303 bp of Le INVINH1. Ten primary transgene (T0) lines were generated. Among them, nine were PCR positive for the transgene based on the PCR analyses.

Transformation of Arabidopsis thaliana with 35S:INVINH1::GFP construct was conducted using particle dropping method. Transient expression of 35S:INVINH1::GFP in onion epidermis was performed by particle bombardment as previously described (Scott et al., 1999).

RT-PCR and Real-Time PCR
Total RNA (1 μg) was extracted from specified tissues and treated with DNase (Promega) and reverse-transcribed using an oligo(dT) primer. Primers specific to INVINH1 for RT-PCR analyses were as follows: 5′-CATATGGAATTTTGATTTTCCT-3′ and 5′-CTCGGAAATTAATTTCTCT-3′. Quantitative real-time RT-PCR analysis was performed using the RotorGene 3000 system (Corbett Research) using the SYBR green detection protocol (TaKaRa). Actin (bt012695) mRNA was used as an internal control, and relative amounts of mRNA were calculated using the comparative threshold cycle method. For analyses of INVINH1 expression, the following primer set was used: 5′-GTGATGTCATGGAGAATTGC-3′ and 5′-GTGATGTCATGGAGAATTGC-3′ (26 and 232 bp downstream of the start codon, respectively). To detect Lin6 transcript, the following primers were used: 5′-GCGGATCCCATTTCTAATGCC-3′ and 5′-GCGGATCCCATTTCTAATGCC-3′. Primers for detecting tomato actin, Lin5, and Lin8 transcripts were generated based on the information described by Fridman and Zamir (2003).

Enzyme Assay and Sugar Measurement
Activities of cell wall, vacuolar, and cytoplasmic invertases and sucrose synthase were assayed as described by Tomlinson et al. (2004). Sugar and starch levels were measured according to Ruan et al. (2003).

In Situ Hybridization
Tomato fruits at 1 DAF were fixed in 4% paraformaldehyde containing 0.1% Tween 20 and 0.1% Triton at 4°C overnight. The samples were dehydrated in a graded ethanol series and transparented in xylene. Thereafter, the samples were infiltrated sequentially with Paraplast Plus (Sigma-Aldrich) at Paraplast:xylene volume ratio of 1:3, 1:1, and 3:1 for 3 h at 60°C at each step. They were then embedded in 100% Paraplast and stored at 4°C.

For making sense and antisense RNA probes, the primer set of Lin5F, 5′-GCGGATCCGAAGTTGAGAATTGC-3′, and Lin5R, 5′-CTCGGATCCGCTTCTAATGCCAC-3′, was used to amplify a 720-bp fragment of Lin5, whereas a 516-bp fragment of INVINH1 was generated using the following primers: INVINH1F, 5′-AAGAATTCGTTCTTCTAATAGTTC-3′, and INVINH1R, 5′-GCGGATCCGAAGTTGAGAATTGC-3′. The fragments were cloned into pBluescript and linearized with BamHI and EcoRI for sense and antisense probes, respectively. Sense and antisense probes were synthesized on T3 and T7 RNA polymerase, respectively, with digoxigenin-UTP as label. Since the transcripts were longer than 500 bp, alkaline degradation was performed to obtain a probe of ~200 bp in length (Cox and Goldberg, 1988).

Sections at 10 μm thickness were cut using a microtome. In situ hybridization was performed as previously described (Xu et al., 2008).

Coimmunoprecipitation
The full-length cDNA of Lin5 without its stop codon was fused with the HA tag sequence (YPYDVPDYA; see Wood et al., 2008). This Lin5-HA fusion construct was digested with SacI and NdeI and cloned into pCAMBIA1300 downstream of the 35S promoter. The 35S:Lin5-HA and 35S::INVINH1-GFP constructs were cotransformed into Arabidopsis. Plants transformed with 35S:LeLin5-HA or 35S::INVINH1-GFP alone were used as controls. Cell wall protein was extracted as described by Pellenc et al. (2004) for coimmunoprecipitation analyses (see below).

The protein-A sepharose (P-AS) beads were prepared following the instructions provided by the manufacturer (Sigma-Aldrich). Briefly, the beads were washed with protein extraction buffer three times before mixing with anti HA or GFP antibodies (Sigma-Aldrich). Approximately 4 μg HA or GFP antibody were mixed with 100 μL P-AS bead solution and incubated at room temperature for 15 min. The antibody bound P-AS beads were then incubated with the plant protein extract at ~5 μg/μL for 2 h at 4°C. The immunoprecipitate was centrifuged at 10,000g for 3 min. The supernatant was discarded. The pellet was resuspended with extraction buffer and centrifuged at 10,000g for 3 min. Proteins bound to the antibody-P-AS beads were released by adding 2× SDS gel loading buffer and boiling for 4 min. Following centrifugation at 10,000g for 5 min, an equal volume (15 μL) of each sample was fractionated by SDS-PAGE. Immunoblotting was performed using anti-HA or anti-GFP antibodies. The protein signals were detected using anti-rabbit, horseradish peroxidase-conjugated secondary antibody as previously described (Ruan et al., 1997).

Protein Gel Blot Analyses
Cell wall protein was isolation as previously described (Bate et al., 2004). The denatured protein samples were separated on 12% SDS–polyacrylamide gels and transferred to an Immobilon-P membrane (Millipore Inter-tech). Thereafter, the membranes were blocked with 5% nonfat dry milk in PBS, pH 7.2, plus 0.1% Tween 20 and then probed for either cell wall invertase using polyclonal anti-maize INCW2 antibodies raised in mice (Carlson and Chourey, 1999) or for INVINH1 using antibodies raised in rabbit against a synthetic peptide of NNNLVEVTC derived from the N terminus of INVINH1 (Mimotopes). Protein signals were detected using goat anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase for cell wall invertase and INVIH1, respectively, followed by visualization using a chemiluminescence system and densitometric quantification using the ImageJ program (http://rsb.info.nih.gov/ij/).

Immunoblotting was performed using anti-HA or anti-GFP antibodies. The anti-INVINH1 and anti-maize INCW2 antibodies detected a polypeptide of 16.4 and 64 kD, respectively, which in turn matched the predicted size of tomato INVINH1 and cell wall invertase (Le Lin5 from fruit and Le Lin6 and 8 from leaf extracts; see Results). As Le Lin5, 6, and 8 are highly homologous to each other with almost identical molecule size (Godd and Roitsch, 1997), the anti-maize INCW2 antibodies recognized all of them if present.

Fluorescent Imaging of CF and Callose
The loading and fluorescent imaging of CF in 1-d-old tomato fruit were conducted according to Ruan et al. (2001). Fluorescent labeling of callose with Aniline Blue was performed as described by Ruan et al. (2004).

Chlorophyll Fluorescence Measurement and ABA Treatment
The ratio of variable to maximal chlorophyll fluorescence (Fv/Fm) was measured as described by Wingler et al. (2004).

For ABA treatment, the second expanded leaves from the top of the plant were treated with ~20 μL of 10−4 M ABA or water once a day for 5 d. The impact on leaf senescence, Fv/Fm ratio, and the apoplastic...
invertase activity was assessed 2 d afterwards. For treatment of seedlings, tomato seeds were first germinated in half-strength MS medium for 10 d. The seedlings were then transferred to either half-strength MS medium containing 10 or 100 μM ABA or half-strength MS only as a control for 24 or 48 h.

Measurement of Cytokinin

Cytokinins were extracted and purified according to Li et al. (2008). Briefly, fresh leaves (0.2 g) were ground to a fine powder in liquid N2 and extracted with precold 80% methanol at 4°C overnight. The supernatant was collected after centrifugation at 3000 g for 10 min. The pellet was reextracted with 80% methanol. The two supernatant fractions were combined and dried in vacuum and resolved in NH4Ac (0.1 M, pH 9.0). After passing through a C18 Sep-pak column, the eluted solution was dried and dissolved in water. The samples were then subjected to reverse-phase C18 HPLC/6520 Accurate-Mass Q-TOF LC/MS analyses (Agilent Technologies), and the UV absorbance was monitored at 269 nm. Analysis was repeated with at least three different samples.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL database under the following accession numbers: Os invinh1, AK288558; Os invinh2, AK070037; Os invinh3, AK070037; Os invinh6, AK110798; ZM CWINVINH1 (see Bate et al., 2004); ZM invinh2, AX214357; ZM invinh3, AX214336; AT invinh, AT1G48010; NT invinh, AY594179; NT CWINVINH, Y12805; INVIN1, AJ010943; Lin5, AJ272304; Lin6, AF506005; Lin8, AF506007; actin, bt012695; SENU2, AJ003137; SENU3, Z48736; SolyCIF, SGN-U332870; Arabidopsis CWINV1, At3g13790; Arabidopsis CWINV4, At2g36190.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Phylogenetic Analysis of Tomato INVINH1 and Other Inhibitors.

Supplemental Figure 2. Overexpression of INVINH1 in Tomato Specifically Inhibited Activity of Cell Wall Invertase (CWINV).

Supplemental Figure 3. ABA-Induced INVINH1 Expression in Tomato Seedlings.

Supplemental Figure 4. Silencing INVINH1 in Tomato Increased Cell Wall Invertase Activity and Delayed ABA-Induced Leaf Senescence.

Supplemental Figure 5. Cytokinin Content in Tomato leaves of Null and INVINH1-RNAi Plants.

Supplemental Figure 6. Sucrose Synthase Activity in Developing Tomato Fruit of Null and INVINH1-RNAi Plants at 1 and 10 DAF.

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Posttranslational Elevation of Cell Wall Invertase Activity by Silencing Its Inhibitor in Tomato Delays Leaf Senescence and Increases Seed Weight and Fruit Hexose Level
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