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Overexpression of yeast spermidine synthase impacts ripening, senescence and decay symptoms in tomato

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SUMMARY

Polyamines (PAs) are ubiquitous, polycationic biogenic amines that are implicated in many biological processes, including plant growth and development, but their precise roles remain to be determined. Most of the previous studies have involved three biogenic amines: putrescine (Put), spermidine (Spd) and spermine (Spm), and their derivatives. We have expressed a yeast spermidine synthase (*ySpdSyn*) gene under constitutive (CaMV35S) and fruit-ripening specific (E8) promoters in *Solanum lycopersicum* (tomato), and determined alterations in tomato vegetative and fruit physiology in transformed lines compared with the control. Constitutive expression of *ySpdSyn* enhanced intracellular levels of Spd in the leaf, and transiently during fruit development, whereas *E8-ySpdSyn* expression led to Spd accumulation early and transiently during fruit ripening. The *ySpdSyn* transgenic fruits had a longer shelf life, reduced shriveling and delayed decay symptom development in comparison with the wild-type (WT) fruits. An increase in shelf life of *ySpdSyn* transgenic fruits was not facilitated by changes in the rate of water loss or ethylene evolution. Additionally, the expression of several cell wall and membrane degradation-related genes in *ySpdSyn* transgenic fruits was not correlated with an extension of shelf life, indicating that the Spd-mediated increase in fruit shelf life is independent of the above factors. Crop maturity, indicated by the percentage of ripening fruits on the vine, was delayed in a *CaMV35S-ySpdSyn* genotype, with fruits accumulating higher levels of the antioxidant lycopene. Notably, whole-plant senescence in the transgenic plants was also delayed compared with WT plants. Together, these results provide evidence for a role of PAs, particularly Spd, in increasing fruit shelf life, probably by reducing post-harvest senescence and decay.

Keywords: polyamines, shelf life, shriveling, transgenic, tomato, fruit ripening.

INTRODUCTION

Post-harvest plant biology encompasses a wide arena of investigative science, ranging from applying growth regulators to delay senescence (and ripening) of the harvested crop, developing controlled atmospheres or plastic covers for storage, to engineering the genetic make-up of the produce (Paliyath and Murr, 2008). Depending on the region of the world, post-harvest losses vary from 10 to 30% in the developed world, to about 70% in some of the developing nations because of the highly perishable nature of these commodities. As the metabolic signals regulating processes involved in post-harvest deterioration of produce quality are not yet well established, progress in reducing post-harvest losses and extending the shelf life of fresh produce has been limited (Paliyath and Murr, 2008). A number of factors contribute to the post-harvest losses of fresh produce and flowers, among which over-ripening (excessive softening), shriveling and microbial decay account for the major maladies. Softening, shriveling, surface cracking and premature senescence of commodities are undesirable features that are thought to be associated with developmentally-regulated metabolic processes. Cell wall modifying enzymes and proteins, namely, polygalacturonase (PG), pectin

methylesterase (PME), β-galactosidase (TBG), xyloglucan endotransglucosylase/hydrolases (XTH) and expansin (EXP) contribute significantly to fruit softening (Brummell, 2006; Saladié *et al.*, 2006; Bennett and Labavitch, 2008; Negi and Handa, 2008). It is also known that several plant growth regulators and their signaling pathways interact to impact these processes (Gan and Amasino, 1997; Mattoo and Handa, 2004; Bennett and Labavitch, 2008). One such major player is the plant hormone ethylene, the control of which results in dramatic changes in ripening and ripening-related fruit softening (Oeller *et al.*, 1991; Giovannoni, 2007; Bennett and Labavitch, 2008).

Growth-promoting and anti-senescence biogenic polyamines (PAs) are important factors in processes such as embryo development, flowering, fruit set, seed production, fruit ripening and senescence (Nambeesan *et al.*, 2008). Commonly found PAs in plants are the diamine putrescine (Put), tri-amine spermidine (Spd), and tetra-amine spermine (Spm). The biosynthesis of Spd and Spm starts with *S*-adenosylmethionine (SAM), which is converted to decarboxylated SAM (dcSAM) by SAM decarboxylase (SAMdc). The production of dcSAM, donor of an amino propyl group, is a rate-limiting step for the synthesis of Spd and Spm (Mehta *et al.*, 2002; Wei Hu *et al.*, 2006). Put is converted to Spd, which is then converted to Spm, by the sequential addition of aminopropyl residues via reactions catalyzed by Spd synthase (SpdSyn) and Spm synthase, respectively (Mehta *et al.*, 2002). Expression of a yeast *SAMdc* gene (*Spe2*; *ySAMdc*) in *Solanum lycopersicum* (tomato) in a fruit-specific manner indicated that SAM availability was not limiting for ethylene production in ripening tomato fruit (Mehta *et al.*, 2002). The consequences of ripening-specific expression of *ySAMdc* in tomato include two or threefold higher lycopene levels, prolonged vine life and improved fruit juice quality (Mehta *et al.*, 2002). In addition to the increased conversion of Put into Spd and Spm, the *ySAMdc* transgenic fruits accumulated other metabolites specifically involved in nitrogen (N) sensing/signaling and N:C interactions, which resulted in higher fructose/sugar and acid/sugar ratios in the red fruit (Mattoo *et al.*, 2006). Transcriptome analyses of these fruits indicated that Spd and Spm function as anabolic growth regulators (Mattoo *et al.*, 2007; Srivastava *et al.*, 2007; Mattoo and Handa, 2008).

S-Adenosylmethionine (SAM) is a common methyl donor for many cellular processes (Martin-Tanguy, 2001). Upregulating *SAMdc* expression in plants may impact the intracellular SAM pool, indirectly leading to the observed phenotypes of the *ySAMdc* transgenic tomato fruits. To gain further insight into the role of higher PAs in fruit, we expressed a yeast *SpdSyn* gene (*ySpdSyn*) under constitutive (CaMV35S) and ripening-induced (E8) promoters, and analyzed vegetative and fruit development in the resulting transgenic tomato plants. Expression of *ySpdSyn* in tomato led to increased levels of Spd but not Spm in the leaf and ripening fruits. Consequently, shriveling and post-harvest decay in fruits, and whole-plant senescence, were retarded in the transgenic plants, thus prolonging their shelf life. These significant changes in the fruit phenotype were not closely associated with changes in the transcript levels of several cell wall-degrading and membrane-modifying enzymes. These data in conjunction with the extensive characterization of *ySAMdc* transgenic tomato fruits suggest multiple roles of PAs in plants.

RESULTS

Yeast SpdSyn expression alters PA levels in tomato leaves

Independently transformed tomato plants harboring the chimeric constructs *CaMV35S-ySpdSyn* or *E8-ySpdSyn* (Figure 1a,b) were selected, and the presence of the introduced gene was analyzed by PCR (Figure 1c). Expression of *ySpdSyn* in leaves from 11 independent *CaMV35S-ySpdSyn* lines and in fruits from four *E8-ySpdSyn* lines was confirmed by RNA-blot and RT-PCR analyses, respectively. Variable levels of the *ySpdSyn* transcript were observed in CaMV35S transgenic plants (Figure 1c, middle panel). Further confirmation of transgene expression was achieved by immunoblot analysis using ySpdSyn polyclonal antibodies, as described in the Experimental procedures. Immunoblot data paralleled the RNA blot data obtained from lines 1, 3, 4, 6, 7, 10 and 15, but no immune-reactive bands were detected in lines 8, 9, 11 and 12, or in the wild type (WT) (Figure 1c, compare the western blot with the RNA blot). Two protein bands were apparent on the immunoblots, one of which could represent a modified form of ectopically expressed ySpdSyn protein. Neither of the two bands represents endogenous SpdSyn protein because no cross-reactive protein was detected in cell extracts isolated from the WT fruit. Although *E8-ySpdSyn* lines did not show detectable levels of ySpdSyn protein, RT-PCR analyses detected *ySpdSyn* transcripts in lines 5, 8, 9 and 17 (Figure 1c, PCR and RT-PCR analyses on the right). Based on the *ySpdSyn* transcript expression data, lines C1, C3, C4 and C15 (with the prefix C representing the constitutive CaMV35S promoter), and lines E8-8 and E8-9 (with the prefix E8 representing the ripening specific-E8 promoter) were selected for generating lines homozygous for the introduced genes (see Experimental procedures for details). In all subsequent experiments transgenic lines homozygous for the introduced gene were used.

Fully expanded leaves from WT and homozygous transgenic lines were analyzed for Put, Spd and Spm content. Compared with the WT, significantly higher levels (51–77%) of Spd were present in all four CaMV35S transgenic lines (Figure 1e), which was associated with a corresponding decrease in Put content (Figure 1d). Surprisingly, Spm content in leaves of the CaMV35S transgenic lines was dramatically lowered compared with that in WT leaves

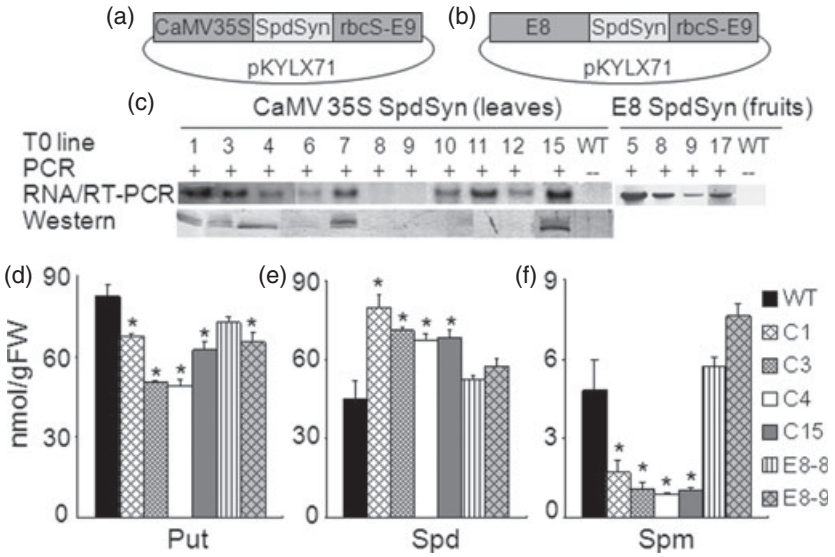

Figure 1. Generation and confirmation of transgenic tomato plants transformed with yeast spermidine synthase.
Chimeric constructs of yeast spermidine synthase (*ySpdSyn*) fused to the CaMV35S promoter (a) or fruit-specific, E8 promoter (b) in the pKYLX71 vector are shown. (c) PCR analysis of transformed lines indicating the presence (+) or absence (–) of the introduced *ySpdSyn* gene (upper panel, PCR). RNA blot of leaf RNA of *CaMV35S-ySpdSyn* lines and RT-PCR analysis of fruit pericarp RNA from *E8-ySpdSyn* lines, respectively (middle panel, northern blot). Immunoblot analysis of leaf proteins from *CaMV35S-ySpdSyn* plants (lower panel, western blot). Levels of putrescine (Put, d), spermidine (Spd, e) and spermine (Spm, f) in fully expanded leaves of transgenic lines, indicated on the right-hand side. Error bars represent the standard error of the means (n = 3); *significant difference from the wild type (α = 0.05).

(Figure 1f). As expected, PA levels in leaf tissue from E8-8 and E8-9, lines homozygous for *ySpdSyn* under the control of the fruit-specific E8 promoter, were not significantly altered, indicating the tissue-specific nature of the E8 promoter. Collectively, these results demonstrated that *ySpdSyn* was functionally active in tomato.

Expression of *ySpdSyn* does not affect endogenous SlSpdSyn expression during fruit development and ripening

Transcript levels of endogenous *SpdSyn* (*SlSpdSyn*) and the introduced *ySpdSyn* were quantified by quantitative RT-PCR during fruit development and ripening in C4, C15, E8-8, E8-9 and WT fruits (Figure 2a,b). In the WT, steady-state levels of *SlSpdSyn* transcripts remained similar from PN7 (post-anthesis + 7 days) to BR3 (breaker + 3 days) stages of fruit development and ripening, but were between two- and threefold higher at later stages (BR7 and BR14) of fruit ripening (Figure 2a). Levels of *SlSpdSyn* transcripts during fruit development and ripening in all transgenic lines examined were similar to WT, indicating that *ySpdSyn* did not induce gene silencing in transgenic tomato fruit (Figure 2a). Under the CaMV35S promoter, the highest levels of the *ySpdSyn* transcripts were found in mature green (MG) fruit, which decreased by about threefold at the onset of fruit ripening, followed by another increase during the later stages of fruit ripening (Figure 2b). Accumulation of *ySpdSyn* transcript under the E8 promoter in E8-8 and E8-9 lines was seen in ripening fruits, with data consistent for the induction of E8 being concomitant with the onset of ripening. Surprisingly, PN14 fruits of the E8-8 line also exhibited *ySpdSyn* transcript accumulation, suggesting some leakiness of this promoter. Accumulation of the *ySpdSyn* transcript driven by the E8 promoter was much lower than the CaMV35S promoter, indicating that it is a weaker promoter.

ySpdSyn expression alters PA levels in tomato fruits

Contents of Put, Spd and Spm were higher in WT fruits at PN7, declined at PN14, increased transiently during the onset of ripening and decreased during later stages of ripening (Figure 2c–e). Significantly higher levels of Spd were present in C4 fruits (23%) at the PN7 stage, and in C15 fruits (28%) at the PN14 stage (Figure 2d). Thus, the accumulation of Spd in C4 and C15 fruits was transiently higher than the WT fruits. Similarly, in the E8-8 fruits, Spd accumulation was higher (36% more) only at the BR stage of ripening. The transient nature of Spd accumulation was not apparent in E8-9 fruits, probably because of the lower expression of *ySpdSyn* in fruits from this line (Figure 2b). Put content was generally lower or similar in fruits from the transgenic genotypes compared with WT fruits, except at BR7, when the Put content was relatively higher, except in E8-9 fruits (Figure 2c). A dramatic decrease in Spm accumulation was found in C4 and C15 fruits during early fruit development, and was undetectable from the BR and BR3 stage onwards, respectively, compared with WT fruits (Figure 2e). In comparison, the Spm accumulation in E8-8 and E8-9 fruits was largely similar to that in the WT (Figure 2e).

Expression of *ySpdSyn* delays developmentally regulated whole-plant senescence

The effects of *ySpdSyn* introduction in transgenic lines on tomato growth and development were evaluated in the field (Figure 3). Plant growth in the WT, measured as vegetative fresh weight, reached a maximum at 110 days after germination (DAG), and declined thereafter (Figure 3). Remarkably, and unlike that in the WT, all transgenic lines continued to gain fresh weight for 17 additional days (137 DAG), which was sustained for a further 34 days (144 DAG) until the plants were harvested (Figure 3). At

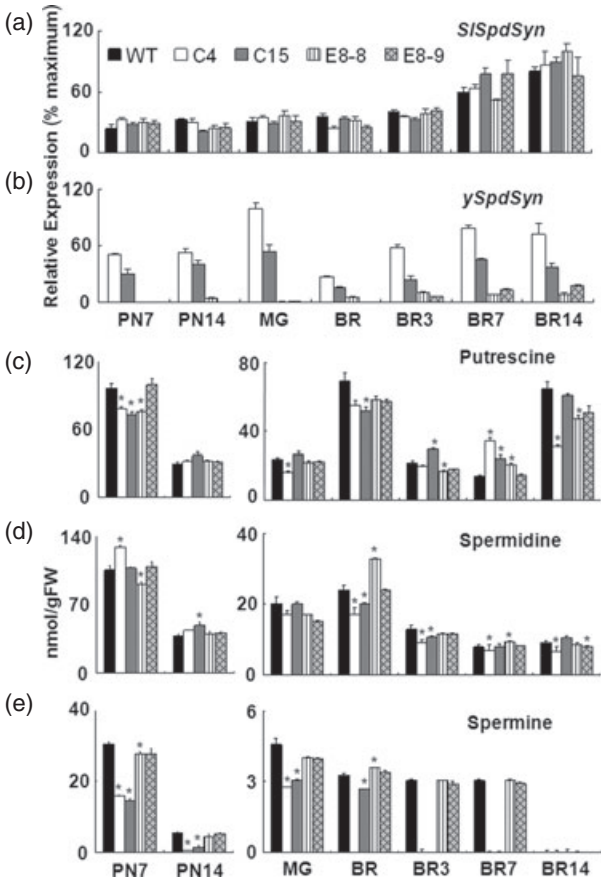

Figure 2. Characterization of transgenic lines expressing the *ySpdSyn* gene.
(a) Quantitative RT-PCR analysis of transcript levels of endogenous spermidine synthase (*SlSpdSyn*) in the fruit from wild type (WT), and homozygous CaMV35S-C4, CaMV35S-C15, E8-8 and E8-9 lines during development and ripening. *SlSpdSyn* expression is shown relative to its expression in WT fruits at PN7 (post-anthesis + 7 days).
(b) Quantitative RT-PCR analysis of transcript levels of the transgene (*ySpdSyn*) in the fruits of homozygous CaMV35S-C4, CaMV35S-C15, E8-8 and E8-9 lines during development and ripening. *ySpdSyn* expression is shown as the percentage of C4-MG fruits that had the maximum level of *ySpdSyn* transcripts in the transgenic tomato fruits.
(c, d and e) Levels of Put (c), Spd (d) and Spm (e) during the development and ripening of fruit from the WT, and from the homozygous transgenic lines described in (a) and (b). Fruit developmental stages: BR, breaker; BR3, breaker + 3 days; BR7, breaker + 7 days; BR14, breaker + 14 days; MG, mature green; PN7, post-anthesis + 7 days; PN14, post-anthesis + 14 days. Polyamine levels were determined from one-third of the blossom end of five fruits per plant, collected from five independent plants for each genotype. Error bars represent the standard error of the means (n = 3). *Significant difference from the wild type at the corresponding stage (α = 0.05).

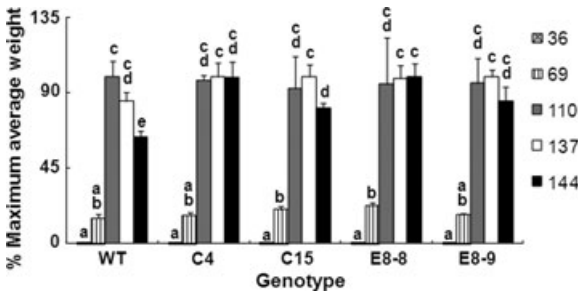

Figure 3. Developmentally-regulated whole-plant senescence is downregulated in *ySpdSyn* transgenic lines.
Above ground plant masses from the wild type (WT), and from homozygous transgenic lines, were harvested by cutting plants above the roots on the indicated days after germination (DAG). Fruits were removed and the remaining vegetative mass was weighed. The vegetative weight for each genotype at the indicated time intervals were averaged. The percentage of maximum weight gain for each plant in the respective genotype during the growing season was determined. This data was statistically analyzed using Duncan's multiple range test. The bars represent means ± SEs. Similar letters above the bars indicate that means were not significantly different at α = 0.05.

harvest, C4 had 42% higher vegetative fresh weight compared with the WT. Fruit set in transgenic lines was similar to that in the WT, except that a significant increase at 110 DAG was observed in the C15 line (Figure S1a). Significant differences were not found in the average fruit weight among the WT and transgenic lines, except for E8-9, which had around 20% lower fruit weight at 144 DAG (Figure S1b). These data suggest that the overexpression of *ySpdSyn* does not affect fruit yield, but significantly delays developmentally regulated vegetative senescence.

Expression of *ySpdSyn* delays the onset of ripening

The effect of *ySpdSyn* expression on fruit ripening was chronologically determined by analyzing the proportion of ripe fruits on field plants. At 110 DAG about 5% of WT fruits were already ripening, in comparison with 0–3% of fruits from the four transgenic lines that had started showing signs of ripening. The trend in these differences was apparent, but because of variations among replicates, the statistical significance was low (Figure 4, inset). However, on 137 DAG significant differences in ripening pattern were clear in the fruit from the C4 line: compared with 45–53% of the fruit from the WT, C15 and E8 lines that had ripened, only 33% of fruits from the C4 line were at the same ripening stage (Figure 4a). By 144 DAG, over 77% of the fruits in all lines were ripe, and no significant difference among the genotypes was observed (Figure 4a). It was clear that *ySpdSyn* expression delayed the onset of ripening in the C4 line.

ySpdSyn expression may enhance fruit lycopene content, but does not affect the rate of ethylene production

Fruit color development was visually different in the transgenic genotypes. Hence, lycopene levels were quantified in red ripe fruits from WT, C4, C15, E8-8 and E8-9 lines. Only red ripe fruits (BR14) from the C4 line had significantly higher (40%) lycopene levels in comparison with the WT and remaining transgenic lines (Figure 4b). This line also had the highest expression of the *ySpdSyn* transgene throughout fruit development and ripening. It is likely that other lines did not reach the threshold levels of PAs to enhance lycopene accumulation to the extent previously observed, where both

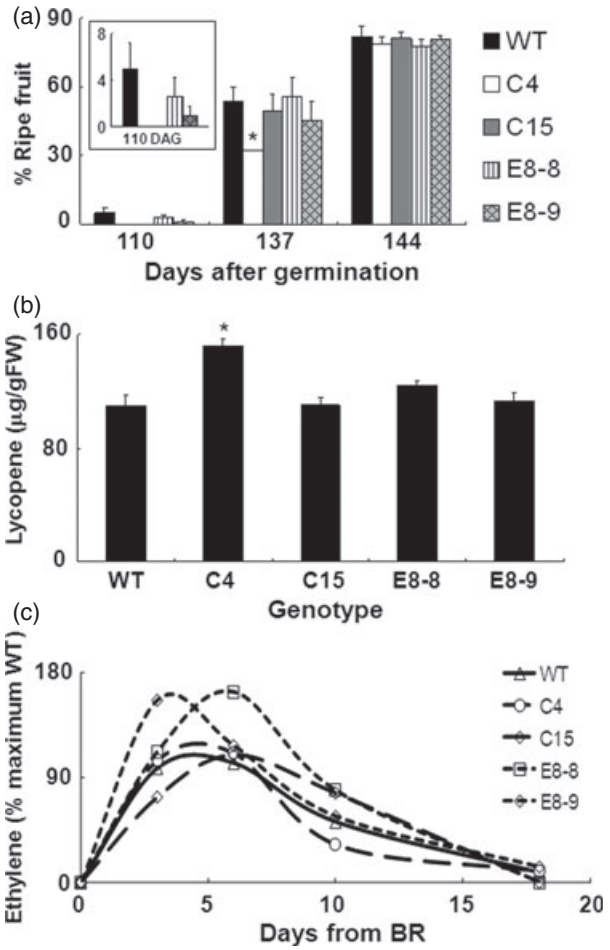

Figure 4. The fruit ripening *on planta*, lycopene accumulation and rate of ethylene production are differentially affected in the homozygous *ySpdSyn* transgenic and wild-type (WT) tomatoes.
(a) Fruit ripening *on planta*: fruits were collected from individual plants, separated into green and ripening/ripe groups, and counted. Green fruits were all green, without any indication of orange/red color at the blossom end. Fruits that were partly or completely red were classified as belonging to the ripening/ripe group. The proportion of green and ripening/ripe fruits at the indicated days after germination (DAG) was determined. The inset shows the percentage of ripening/ripe fruit at 110 DAG in all five genotypes tested.
(b) Lycopene content: one-third of the blossom end of fruits from the breaker + 14 days (BR14) stage was used for lycopene content determination, as described in Experimental procedures. Error bars represent the standard error of the means; *significant difference from the wild type ($n = 5$; $\alpha = 0.05$).
(c) Ethylene production: breaker fruits were harvested from each genotype and at the indicated time intervals the rate of ethylene production was measured using gas chromatography.

Spd and Spm accumulated because of the transformation with the *ySAMdc* gene (Mehta *et al.*, 2002). Fruits from the WT, C4 and C15 lines did not show significant differences in the rate of ethylene production (Figure 4c). The rate of ethylene production in E8-8 and E8-9 fruits was higher, and peaked 1–2 days later in E8-8 than in the remaining genotypes analyzed (Figure 4c).

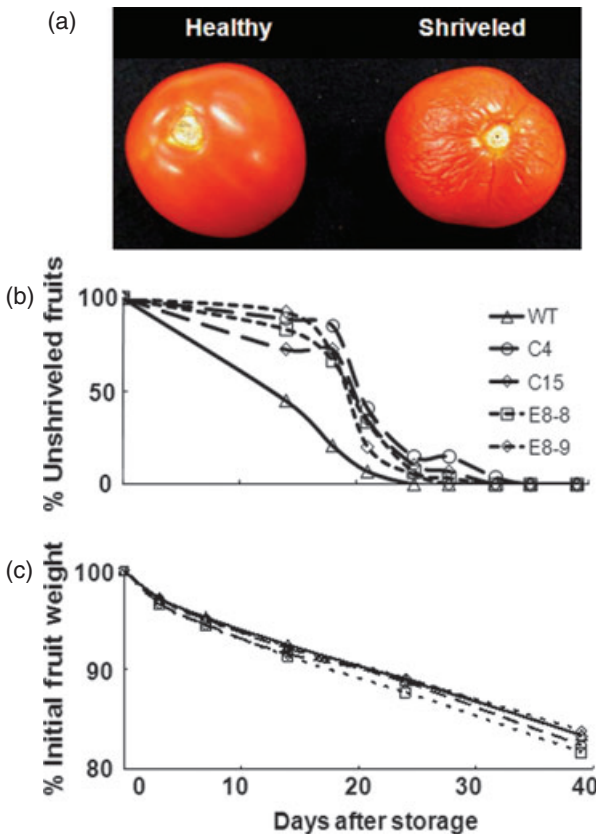

Figure 5. The prolonged shelf life and the delay in the development of shriveling symptoms during storage of homozygous *ySpdSyn* transgenic lines were independent of water loss.
(a) Healthy and shriveled phenotypes. Fruits from each genotype were harvested and stored at 25 ± 2°C ($n \geq 50$). The fruit were evaluated at the indicated time intervals for shelf life/shriveling (b) and fresh weight loss (c) during storage. The average fresh fruit weight at the time of harvest for the wild type (WT), and for the C4, C15, E8-8 and E8-9 lines, were 62 ± 4, 69 ± 12, 56 ± 6, 64 ± 12 and 57 ± 5 g, respectively.

Transgenic *ySpdSyn* fruits have a longer shelf life and delayed decay symptoms during storage

The delay in vegetative senescence and fruit ripening in field experiments prompted us to further examine the effect of *ySpdSyn* expression on shelf life (Figure 5a,b) and decay development (Figure 6a,b) in fully ripened fruits from homozygous C4, C15, E8-8, E8-9 and WT lines. Fully red fruits ($n \geq 50$) were harvested and held at 25 ± 2°C in the laboratory. Post-harvest fruit shriveling was significantly delayed in transgenic fruits compared with WT fruits (Figure 5b). The time to post-harvest shriveling of 50% fruits (TPS_{50}) was 13 days for WT fruits and about 21 days for fruits from the four transgenic lines. Within the transgenic lines, fruits from the C4 line were the most resistant to shriveling (Figure 5b). Delayed shriveling of transgenic fruits was not related to the rate of water loss, as all of the genotypes tested had a weight loss pattern

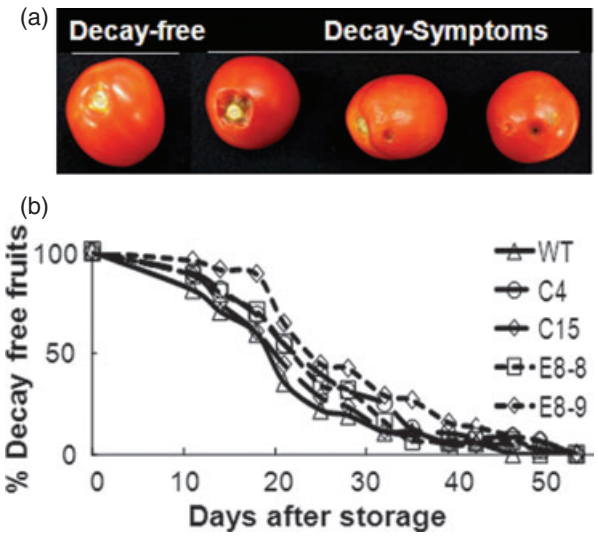

Figure 6. Decay symptom development during post-harvest storage of fruits from wild-type (WT) and homozygous transgenic (C4, C15, E8-8 and E8-9) plants.
(a) Fruits without and with decay symptoms, respectively. Red fruits from each genotype ($n \geq 50$) were stored at 25 ± 2°C, and were evaluated for decay symptom development at the indicated time intervals (b).

similar to that of the WT fruits (Figure 5c). In fact, C4 and E8-8 fruits registered slightly higher rates of water loss (0.43 and 0.44 g day^{-1}) compared with C15, E8-9 and WT fruits (0.4, 0.39 and 0.4 g day^{-1}, respectively), further indicating that reduced water loss is not the cause for the longer shelf life and resistance to shriveling of the transgenic fruits.

We evaluated the rate of development of post-harvest decay/rot symptoms during storage of transgenic and WT fruits (Figure 6a,b). The time for post-harvest decay development on 50% of stored fruits (TPD_{50}) was approximately 23 days for C4, E8-8 and E8-9 fruits, and 20 days for WT and C15 fruits (Figure 6b). Decay symptoms consisted of small circular depressions on the surface of the ripe tomato fruit, which are typical of anthracnose caused by the fungus *Colletotrichum coccodes*. However, the presence of this fungus could not be conclusively determined by the Plant and Pest Diagnostic Laboratory at Purdue University. The fungus that was consistently isolated from the stored fruits was *Alternaria alternata*, a post-harvest rot pathogen. Taken together, these results indicate that ectopic expression of *ySpdSyn* delays post-harvest senescence and shriveling, and has a relatively positive although smaller effect on delaying the onset of decay symptoms caused by field pathogens.

Extended shelf life of *ySpdSyn* fruits is not mediated by changes in the expression of cell wall and membrane degradation-related genes

To determine if the observed effects of *ySpdSyn* expression on fruit shriveling and decay development were related to changes in cell wall and membrane disassembly and degradation, we quantified the transcript levels of a range of genes encoding proteins implicated in these processes. The following genes were selected for transcript quantification: polygalacturonase (*SlPG*), pectin methylesterase (*SlPME*), expansins (*SlEXP1* and *SlEXP3*), β-galactosidase 4 (*SlTBG4*), α-xyloglucan endotransglucosylase/hydrolase (*SlXTH5*), lipoxygenase (*SlLOXB*) and phospholipase D (*SlPLDα*). Increased levels of *SlPG*, *SlEXP1*, *SlTBG4* and *SlXTH5* transcripts were apparent during the late stages of ripening in the transgenic lines in comparison with the WT (Figure 7). Although the expression of *SlLOXB* and *SlPLDα*, genes associated with membrane degradation, in the transgenic lines was different from that in the WT, these alterations in expression were not correlated with any delayed shriveling in *ySpdSyn* fruits. *SlXTH5* transcripts showed a roughly twofold increase in C4, C15 and E8-9 fruits at the BR7 stage, but its expression was not detected in any of the E8-8 fruit tested (Figure 7). It may be possible, but has not yet been tested, that the introduced transgene was lodged near or within the *SlXTH* locus, eliminating its expression in E8-8.

DISCUSSION

We have demonstrated here that the introduction of a heterologous yeast spermidine synthase gene (*ySpdSyn*) in tomato causes specific but transient accumulation of the higher polyamine Spd, and extends the post-harvest shelf life of tomato fruits without impairing the ripening process. More significantly, the *ySpdSyn* transgenic fruits had reduced shriveling and decay symptom development. These results bring to the fore previously unreported connections between the introduction of the *SpdSyn* gene and shriveling, ripening, decay symptom development and fruit quality.

Tomato genotypes with extended shelf life include mutants impaired in ripening (*nor*, *alcobaça*, *rin*, *Nr*, *colorless*, *nonripening* and *firme*), transgenic plants altered in the biosynthesis or perception of the ripening hormone ethylene (Tigchelaar *et al.*, 1973; Mutschler, 1984; Brummell and Harpster, 2001; Schuelter *et al.*, 2002; Giovannoni, 2004; Negi and Handa, 2008), transgenic plants with downregulated expression of *LeRab11a*, GTPase (Lu *et al.*, 2001) or *N*-glycans (Meli *et al.*, 2010), and genotypes with altered cuticle architecture resulting in 'delayed fruit deterioration' (DFD; Saladié *et al.*, 2007). Mutant genotypes are generally of lower quality because of the pleiotropic nature, and associated alteration in temporal regulation, of many ripening-related processes, including climacteric respiration and ethylene production (Kopeliovitch *et al.*, 1982). Fruits of transgenic plants in which the production or perception of ethylene is impaired lose coordination between ethylene-dependent and -independent processes, and thus impact fruit quality (Giovannoni, 2002; Klee and Clark, 2002). In the same light, the prolonged shelf life of fruit from transgenic

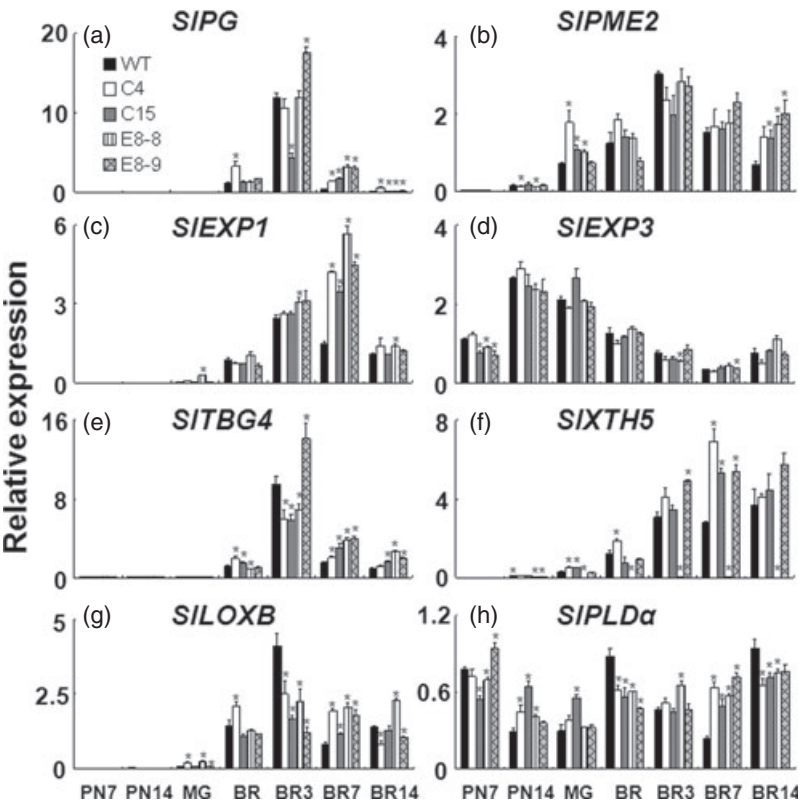

Figure 7. Developmentally regulated expression of cell wall and membrane metabolism-related genes in wild type and homozygous transgenic fruits.
Quantitative RT-PCR analysis was used to quantify the transcript levels of *SlPG* (a), *SlPME2* (b), *SlEXP1* (c), *SlEXP3* (d), *SlTBG4* (e), *SlXTH5* (f), *SlLOXB* (g) and *SlPLDα* (h). The relative expression was calculated using the $\Delta\Delta C_t$ method using *actin* as the reference gene. Error bars represent the standard errors of the means ($n = 3$). *Significant differences from the wild type at the corresponding stage of development ($\alpha = 0.05$). Fruit developmental stages are as shown in Figure 2.

antisense *LeRab11a* lines with full color development was associated with undesirable phenotypes, including determinate growth, reduced apical dominance, branched inflorescences, abnormal floral structure and ectopic shoots on the leaves (Lu *et al.*, 2001). Fruits from the DFD genotype that have slower water loss because of modified cuticles, ripened slowly, taking additional time to develop full color (Saladié *et al.*, 2007). A different strategy used to enhance shelf life while keeping other parameters unaffected in the *N*-glycan-silenced tomato remains to be tested in the field (Meli *et al.*, 2010). Considering the above observations, *ySpdSyn* transgenic lines provide a promising system to positively impact fruit characteristics without yield loss, and provide a new resource to understand the physiology of fruit ripening and senescence in tomato.

The *ySpdSyn* transgenic fruits, like those from DFD and *LeRab11a* antisense plants, undergo full ripening and have an extended shelf life. However, there are important differences. For instance, in *ySpdSyn* fruits, the period for completion of ripening from the BR stage was similar to that in WT fruits. The fruit from DFD plants required an additional 7 days to complete the ripening process in relation to control fruits (Saladié *et al.*, 2007). At the level of gene expression, a twofold decrease in the transcript levels of pectinesterase and polygalacturonase was found in *LeRab11a* antisense fruits (Lu *et al.*, 2001). Such a decrease was not observed in the *ySpdSyn* fruits (Figure 7). Our data indicate that the alteration of PA levels in *ySpdSyn* fruits did not affect the progression of ripening, unlike that observed with many of the mutant genotypes with an altered shelf life. It is possible that the extension of shelf life in *ySpdSyn* transgenic fruits is uncoupled from ripening, as was observed in the DFD and the *LeRab11a* antisense genotypes (Lu *et al.*, 2001; Saladié *et al.*, 2007). These studies are consistent with our current understanding that fruit ripening and senescence represent complex processes co-coordinated by multiple pathways, at least one of which includes regulation by PAs.

A number of genes that seem associated with cell wall loosening and membrane degradation are also implicated in affecting fruit quality, texture, shelf life and pathogen-induced decay (Brummell and Harpster, 2001; Smith *et al.*, 2002; Cantu *et al.*, 2008; Negi and Handa, 2008). Likewise, it is thought that shriveling and a loss of firmness in tomato fruits involves changes in cell wall/membrane composition (Brummell, 2006; Negi and Handa, 2008). However, the decrease in shriveling of *ySpdSyn* transgenic fruits was accompanied by increased levels of *SlPG*, *SlEXP1*, *SlTBG4* and *SlXTH5* transcripts at later stages of fruit development (BR7 and BR14). Also, the expression of *SlLOXB* and *SlPLDα*, genes associated with membrane degradation, was not correlated with delayed shriveling in *ySpdSyn* fruits. Thus the levels of these gene transcripts seem uncoupled from processes that extend fruit shelf life in *ySpdSyn* fruits, observations consistent with findings on DFD fruits (Saladié *et al.*, 2007).

The delayed shriveling of *ySpdSyn* fruits was not associated with reduced water loss, suggesting that mechanisms regulating fruit water loss are not involved in the extension of shelf life in *ySpdSyn* fruits. Interestingly, the downregulation of *PLDα* (Pinhero *et al.*, 2003) and deoxyhypusine synthase (*DHS*; Wang *et al.*, 2005) genes in tomato was associated with firmer fruits than the controls, and in neither case were the differences related to a loss of fresh weight. Hence, an alternative mechanism(s) independent of cell wall/membrane modification and water loss is likely to be involved in mediating the extension of shelf life or shriveling phenomenon in the *ySpdSyn* fruits. Possible mechanisms include direct stabilization of fruit cell wall/membrane by Spd and other PAs, or Spd-mediated extension of a metabolically active state in *ySpdSyn* fruits. That higher PAs induce a highly active metabolic state in tomato fruit has been demonstrated previously (Mattoo *et al.*, 2006; Mattoo and Handa, 2008). Further research is required to assess these and other possibilities.

Expression of *ySpdSyn* also delayed the senescence of vegetative parts of tomato plants. Delayed senescence of E8-8 and E8-9 plants may be attributed to either mobilization of PAs from ripening fruits or leakiness of the ethylene-regulated E8 promoter (Kneissl and Deikman, 1996). Impairment of whole-plant senescence has implications in extending the shelf life of leafy vegetables that are prone to post-harvest desiccation and shriveling, especially at high temperatures. Many studies have shown that the application of PAs delays senescence and aging in plants (Galston and Kaur-Sawhney, 1987, 1995; Mattoo and White, 1991; Lester, 2000; Serrano *et al.*, 2003; Mattoo and Handa, 2004). Exposure to 0.1–1.0 mM Spm for 60 min was enough to prevent the development of senescence in excised, peeled, oat leaf segments (Galston and Sawhney, 1990). Spd (10 mM) treatment delayed senescence of cut carnation flowers (Tassoni *et al.*, 2006). Our *in vivo* results on delayed senescence in the vegetative parts are consistent with these *in vitro* studies, and provide genetic evidence in support of an important role of higher PAs in regulating plant senescence. Programmed plant senescence is greatly influenced by phytohormones, including ethylene, abscisic acid, cytokinin, gibberellins and brassinosteroids (Grbic and Bleecker, 1995; Jordi *et al.*, 1995; Weaver *et al.*, 1998; Sasse, 2003; Mattoo and Handa, 2004; Lim and Nam, 2005; Kim *et al.*, 2006; To and Kieber, 2008). PAs such as Spd may facilitate a delay in whole-plant senescence either directly or through crosstalk with the above phytohormones. Elevated levels of Spd in vegetative plant parts such as the leaves may result in the promotion and/or maintenance of growth, resulting in delayed plant senescence. Such an association of Spd with anabolic processes has been observed in tomato fruits (Handa and Mattoo, 2010).

It is becoming apparent from molecular analysis of mutant genotypes including genetically engineered crops that PAs play significant roles in processes such as chromatin condensation, stability and function of DNA structure, RNA processing, translation, and protein activation (Hyvönen *et al.*, 2007; Mattoo and Handa, 2008; Nambeesan *et al.*, 2008; Mattoo *et al.*, 2009; Pegg, 2009; Handa and Mattoo, 2010). Spm function is linked to salt (Krishnamurthy and Bhagwat, 1989; Yamaguchi *et al.*, 2006; Kusano *et al.*, 2007; Tassoni *et al.*, 2008) and drought (Capell *et al.*, 2004; Yamaguchi *et al.*, 2007) tolerance in plants. Its related form thermospermine (tSpm) is important for stem elongation (Kakehi *et al.*, 2008). The thicker vein (*tkr*; vascular tissue differentiation) phenotype has been linked to Spm synthase (ACL5/TKV) in Arabidopsis (Hanzawa *et al.*, 2000; Clay and Nelson, 2005). PAs also participate in the translational frame shift of at least two known genes via a novel RNA-decoding mechanism (Clare *et al.*, 1988; Balasundaram *et al.*, 1994). Elevated Spd and Spm levels in *ySAMdc* fruits influence steady-state levels of a large number of transcripts, proteins and metabolites in ripening tomato fruits (Mattoo *et al.*, 2006, 2007; Srivastava *et al.*, 2007). It was shown that they enhance metabolic activity even in a fully ripened fruit, a developmentally terminal organ, as evident by the revival of cellular programs underlying N:C signaling, and energy and glucose metabolism (Mattoo *et al.*, 2006; Mattoo and Handa, 2008). Also, the catabolism of PAs by polyamine oxidases leads to the generation of H_2O_2, which is considered to be a second messenger for a number of plant defense responses, including programmed cell death and abiotic stress responses (Cona *et al.*, 2006). Based on a correlation between the levels of PAs and changes in various physiological and biochemical parameters in ripening tomato fruit, it seems that the mechanism of action of the diamine Put differs from that of Spd and Spm (Mattoo *et al.*, 2009; Handa and Mattoo, 2010). Whereas Put is predominately associated with catabolic processes, Spd and Spm show positive correlation with anabolic processes. In light of these observations, it is proposed here that the enhanced shelf life of fruits and delayed senescence of *ySpdSyn* transgenic plants could be a result of the growth-promoting effects of Spd. Although Spd levels were not dramatically high in these genotypes, similar 1.3–2-fold increases were observed in transgenic plants such as Arabidopsis (Kasukabe *et al.*, 2004), rice (Peremarti *et al.*, 2009) and pear (Wen *et al.*, 2008) overexpressing *SpdSyn* and *SAMdc* genes. These studies suggest that a small increment in Spd levels may be sufficient to induce significant physiological changes. Thus, a certain intracellular threshold of Spd may result in slowing down the deterioration of fruit ultrastructure, improving and maintaining fruit texture, and delaying the development of decay symptoms.

EXPERIMENTAL PROCEDURES

Generation of transgenic plants expressing *ySpdSyn*

Complete coding region of *SPE3*, *Saccharomyces cerevisiae* spermidine synthase (*ySpdSyn*; gene ID 856182), was amplified from yeast genomic DNA using the forward primer ScSpe3XhoF (5′-GCCGCTCGAGATGGCACAAGAAATCACTCACCCAA-3′) and the reverse primer ScSpe3XbaR (5′-GCCGTCTAGACTAATTTAATTCC TTGGCTGCCCAG-3′). The amplified 882-bp product was cloned into the pGEM-T Easy vector system (Promega, http://www.promega.com). The SPE3 insert was excised by *Xho1* and *Xba*I, and cloned in the sense orientation between a CaMV35S promoter and the 3′ end of a *Pisum sativum* (pea) *rbcS-E9* gene in pKYLX71 (Schardl *et al.*, 1987), and was designated as *CaMV35S-ySpdSyn* (Figure 1a). The chimeric construct with the E8 promoter, designated *E8-ySpdSyn* (Figure 1b), was generated by replacing the CaMV35S promoter by inserting the 2-kb E8 5′ flanking region, isolated from the pE8mutRN2 plasmid (Giovannoni *et al.*, 1989). The E8 promoter was excised from the pE8mutRN2 plasmid using restriction enzymes *Eco*R1 and *Hind*III, and cloned into pKYLX71 digested with the same restriction endonucleases to remove the CaMV35S promoter. The SPE3 insert was subsequently cloned in the sense orientation between the E8 promoter and the 3′ end of a pea *rbcS-E9* gene in the pKYLX71 vector using *Sac*I and *Xho*I endonucleases. These chimeric constructs were introduced individually into disarmed *Agrobacterium tumefaciens* LBA4404 by chemical transformation, and were then utilized to transform cotyledons of tomato cv. Ohio 8245 (Tieman *et al.*, 1992; Mehta *et al.*, 2002). Fifteen independent transgenic plants expressing *CaMV35S-ySpdSyn* and 17 independent transgenic plants expressing *E8-ySpdSyn* were propagated. The presence of an inserted transgene in the *CaMV35S-ySpdSyn* lines was confirmed by PCR using a forward primer specific to the vector (35SP, 5′-GATGACGCACAATCCCACTATC-3′) and a reverse primer specific to the transgene (*ySpdSyn*, ScSpe3R, 5′-CATTTCTTGGTAGGCAAATTCATCCC-3′). For *E8-ySpdSyn* transgenic lines, an E8-specific forward primer (E8Fend, 5′-GACGTATTGGG TTTCATATTTTAAAAAGGG-3′) and the ScSpe3R primer were used. The expression of *CaMV35S* and *E8-ySpdSyn* was determined by RNA blot and semi-quantitative RT-PCR analyses of leaf and fruit pericarp tissues, respectively, as described below. The ySpdSyn protein in the leaves of *CaMV35S-ySpdSyn* transgenic plants was assayed immunologically (Tieman *et al.*, 1992).

Development of tomato lines homozygous for the *ySpdSyn* transgene

Based on the transgene transcript and protein levels in T_0 lines, four independent transgenic plants expressing *CaMV35S-ySpdSyn* and two transgenic plants expressing *E8-ySpdSyn* were selected. Transgenic plants were selfed, and segregating T_1 seeds were collected. Seeds from a minimum of 20 segregating T_1 seedlings for each selected transgenic lines were collected separately. A minimum of 20 T_2 progeny from each T_1 line were characterized by PCR to identify progenies homozygous for the transgene. The segregants lacking the transgene were classified as azygous lines. In all subsequent experiments, only those lines homozygous for the transgenes and a WT control were used to evaluate the role of *ySpdSyn* expression in tomato. Independent homozygous lines were designated as C1, C3, C4 and C15 for *CaMV35S-ySpdSyn*, and E8-8 and E8-9 for *E8-ySpdSyn* chimeric constructs, respectively.

Field evaluation of *ySpdSyn* transgenic lines

The physiological performance of independent homozygous transgenic C4, C15, E8-8 and E8-9 lines, and the parental WT plants, was investigated in a randomized block design at the Meigs Horticulture Research Farm, Lafayette, Indiana (Tieman *et al.*, 1995). Seeds were germinated in the glasshouse using a compost soil mix with a 16-h day/8-h night photoperiod and day/night temperatures of 23/18°C. Ten 6-week-old glasshouse-grown seedlings from each tomato genotype were transplanted in three randomized blocks. Standard fertilization, weeding and plant protection operations were carried out during the study (Tieman *et al.*, 1995).

Quantification of vegetative growth and fruit yield

Randomly selected plants were harvested by cutting at the ground level on the indicated DAG. Fruits were separated, and fruit and vegetative parts were weighed separately. Fruit set was evaluated by counting the number of fruits per plant. The average fruit weight was calculated by dividing the total fruit weight by the total fruit number. Ripening was determined as the proportion of ripe and green fruit on each harvested plant (Tieman *et al.*, 1995).

Plant material for polyamine and gene expression analyses

Fruits were harvested at PN7, PN14, MG, BR, BR3, BR7 and BR14. Breaker stage fruits were selected based on the development of an initial red coloration at the blossom end of the fruit. Pericarp and gel from one-third of the blossom end of the fruit were collected from five independent plants for each genotype (five fruits per plant). Fruits at PN7, PN14, MG and BR stages were collected from the field plants. BR fruits were held in the laboratory at 25 ± 2°C and samples BR3, BR7 and BR14 were collected for analysis. All the harvested samples were immediately frozen and stored at −80°C until further use.

RNA blot analysis

Total RNA was extracted from leaf tissue of putative *CaMV35S-ySpdSyn* transgenic plants as described by Chomczynski and Sacchi (1987). RNA was fractionated on 1.2% formaldehyde agarose gels, blotted onto Hybond N^+ nylon membranes (Amersham Pharmacia Biotech, now GE Healthcare, http://www.gelifesciences.com) and hybridized with a ^{32}P-labeled probe obtained by random priming (DECAprime™II; Ambion, http://www.ambion.com; Ramakrishna *et al.*, 2003). Quantification of hybridization signals was carried out with a Typhoon 8600 PhosphorImager (GE Healthcare, http://www.gelifesciences.com; Srivastava *et al.*, 2007).

Quantitative RT-PCR analyses

A Stratagene Mx3005P real-time PCR system was used for quantitative qRT-PCR analyses (Stratagene, now part of Agilent Technologies, http://www.agilent.com), and all primers were validated to facilitate the $\Delta\Delta C_t$ method for gene expression analysis (Livak and Schmittgen, 2001). Primer sequences listed from 5′ to 3′ are listed in Table S1. The *SlActin* gene was used to normalize the expression of target genes. Diluted cDNA (1 μl) was used in a 14/15 μl reaction mixture along with the SYBR green-PCR master mix (2X; Applied Biosystems, http://www.appliedbiosystems.com). Melting curve analysis was performed after the PCR reaction to determine the specificity of the PCR products. PCR reactions were performed with the following program: 95°C (10 min); 95°C for 30 sec and 60°C for 1 min, for 40 cycles. At least three replicates were analyzed for quantification of gene expression. Accession numbers for the genes selected for transcript quantification are: endogeneous *SpdSyn* (*SlSpdSyn*, AJ006414), polygalacturonase (*SlPG*, X05656), pectin methylesterase (*SlPME*, U70675), expansins (*SlEXP1*, U82123; *SlEXP3*, AF059487), β-galactosidase 4 (*SlTBG4*, AF020390), α-xyloglucan endotransglucosylase/hydrolase (*SlXTH5*, AY497475),

lipoxygenase (*SlLOXB*, U09025), phospholipase D (*SlPLDα*, AF201661) and actin (*SlActin*, BT012695).

Immunoblot analyses

Polyclonal antibodies were raised against the recombinant *ySpdSyn* protein (Ramakrishna *et al.*, 2003). As the *ySpdSyn* gene does not contain any intron, its full-length coding region was amplified from yeast genomic DNA using a forward primer (ScSpe3BamH1F, 5′-GCCGGGATCCATGGCACAAGAAATCACTCACCCAA-3′) and a reverse primer (ScSpe3Xho1R, 5′-GCCGCTCGAGCTAATTTAATTCCTTGGCTGCCCAG-3′) containing *Bam*H1 and *Xho*I restriction sites, respectively, for cloning into the *Bam*H1- and *Xho*1-digested *Escherichia coli* expression vector pET28A (Invitrogen, http://www.invitrogen.com). The resulting recombinant plasmid was sequenced to confirm the sequence identity of *ySpdSyn*, designated as pET28A-ySpdSyn, and was then expressed in *E. coli* BL21 (DE3) following Invitrogen's recommendations. The recombinant ySpdSyn protein was purified using a nickel NTA column following the manufacturer's instructions (Handbook for Protein Purification; Qiagen, http://www.qiagen.com), and polyclonal antibodies against it were produced in rabbits (Harlan, http://www.harlan.com). The antibodies identified a 40-kDa (expected size) protein band on immunoblots. These antibodies were used to confirm protein accumulation in the leaves of transgenic plants as described by Tieman *et al.* (1992).

Analysis of lycopene content

We used BR14 fruits for lycopene analysis. Frozen pericarp tissue (2 g) was ground in liquid nitrogen, extracted in 5:4 hexane:acetone (v/v), vigorously mixed and centrifuged. The upper hexane layer was collected. Samples were re-extracted five times with the solvent until the pellet was colorless. All collected hexane layers were pooled, 2 ml of 12% NaCl was added to each pool, the contents were mixed and then centrifuged. The hexane layer was collected, diluted 10 times and absorbance was spectrophotometrically determined at 503 nm (Handa *et al.*, 1985).

Ethylene measurement

Individual fruits were placed in air-tight jars for 1 h, and ethylene released into the headspace was quantified by gas chromatography (Tieman *et al.*, 1992; Mehta *et al.*, 2002).

Post-harvest shelf life

Red ripe fruits harvested from the field were stored at 25 ± 2°C on the laboratory bench ($n \geq 50$). At indicated intervals, each fruit was evaluated for signs of shriveling and symptoms of post-harvest rot/decay.

Quantification of polyamine content

Frozen leaf or fruit samples were ground in liquid N_2 and re-suspended in 800 μl of 5% (w/v) cold perchloric acid (PCA) per 0.2 g of ground tissue (Minocha *et al.*, 1994). After mixing, the samples were centrifuged at 20 000 ***g*** for 30 min. As an internal standard, 20 μl of 0.1 mM 1,7-diaminoheptane was added to 100 μl of the supernatant, and the sample was dansylated with 100 μl of 20 mg ml^{-1} dansyl chloride (in acetone) in the presence of 100 μl of saturated sodium carbonate at 60°C for 1 h. The reaction was terminated by the addition of 50 μl of 20 mg ml^{-1} L-asparagine at 60°C for 30 min. Acetone was removed by placing samples in a SpeedVac for 30 min, toluene (400 μl) was added to the sample, and was mixed and centrifuged. The toluene layer (200 μl) was removed and dried under a stream of air. Methanol (1 ml) was added to the sample, mixed for 1 min, and then 50 μl of the sample was filtered through a 0.45-μm filter (National Scientific, http://www.nationalscientific.com). The filtered samples were injected into a Waters HPLC system consisting of two model 510 pumps and a model 715 WISP autosampler. Dansylated PAs were separated using a reverse-phase Xterra C18 (3.8 × 100 mm) column, and then detected on a Hewlett Packard model 1046A fluorescence detector (340- and 510-nm excitation and emission wavelengths, respectively; Hewlett Packard, http://www.chem.agilent.com). A binary gradient composed of solvent A (100% acetonitrile) and solvent B [heptanesulfonate (10 mM, pH 3.4) : acetonitrile (90:10)] was used. A flow rate of 1.1 ml min^{-1} and a gradient elution were used. Initial conditions were set at 50:50 (A:B), followed by a linear gradient as follows: 80:20 (A:B) at 2 min; 100:0 (A:B) at 9 min; 80:20 (A:B) at 12 min; and back to 50:50 (A:B) at 20 min. Authentic standard PAs (Sigma-Aldrich, http://www.sigmaaldrich.com) were extracted and analyzed as described above to determine recovery, and to generate calibration multilevel curves. Integration of peaks was performed for quantification.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Fruit set and fruit weight are not impacted in *ySpdSyn* transgenic plants.

Table S1. Primers used for quantitative and semi-quantitative RT-PCR.

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