A physical, enzymatic, and genetic characterization of perturbations in the seeds of the brownseed tomato mutants

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Abstract

The brownseed mutants (bs1, bs2, and bs4) of tomato all possess dark testae and deleteriously affect seed germination speed and/or final percentage. Poor germination performance of the bs1 but not the bs4 mutant, was due to greater impediment to radicle egress. Testa toughening (bs1) was prevented by drying in N2. However, poor germination speed was hardly affected by drying. GA4+7 did not ameliorate germination percentage or speed (bs1, bs2), whereas bs4 seeds commenced radicle protrusion sooner and had a greater germination percentage. bs1 mutant seeds have two times more catalase activity while those of bs4 contained six times more peroxidase and almost two times more catalase activity than WTs. bs4 release only half of the reactive oxygen species into the media than WT during imbibition. EPR detected the presence of free radicals in bs1 and its WT. bs mutants were epistatic to 12 anthocyaninless mutations, at least some of which produce seeds of lighter than usual testa colour. Macro-arrays of subtractive, suppressive PCR products identified differentially regulated transcripts between seeds of bs4 and WT. EST identity suggests bs4 does not exit the developmental programme upon attaining maturity.

Key words: Catalase, electron paramagnetic resonance (EPR), free radicals, germination, Lycopersicin esculentum, macro-array, peroxidase, seed, subtractive-suppressive PCR, testa.

Introduction

The enclosure of the embryo and endosperm in integuments of strictly maternal origin (testa, pericarp) has led to interactions among tissues of different parental genetic contribution to establish seed longevity and control of radicle protrusion (Koornneef and Karssen, 1994; Léon-Kloosterziel et al., 1994; Debeaujon et al., 2000). Studies of arabidopsis and tomato mutants deficient in testa pigmentation (aberrations in the shikimic acid pathway) have underscored the importance of the testa (Atanassova et al., 1997a, b; 2001; Debeaujon and Koornneef, 2000; Downie et al., 2003b) in these processes. However, understanding of the testa attributes that confer high quality performance in such diverse functions remains poor (Koornneef et al., 2002). Nevertheless, transparent testa (tt; arabidopsis) and anthocyaninless (tomato) mutants have shown that control of radicle protrusion is exerted through the deposition of shikimate metabolites in the testa during development (Debeaujon et al., 2000; Atanassova et al., 1997a). Derivatives of the shikimate pathway have been found to crosslink extensin, cellulose, pectin, and other components of the cell wall. The assembly of the various members of the shikimate family lead to a diversity of polymers that have long been associated with increasing structural strength, providing defence against pathogens, and decreasing water permeability, attributes that are consistent with the final role of the testa (Mohamed-Yaseen et al., 1994).

The polymerization of shikimate metabolites is due to endogenous oxidative enzymatic action during testa mat-
uration (Gillikin and Graham, 1991) coupled with an alteration in the physical environment surrounding the propagule. These changes profoundly alter testa permeability, oxygen availability to the embryo following imbibition, and/or testa physical strength (Egley et al., 1983; Gillikin and Graham, 1991; Qi et al., 1993). Studies using ethylene-insensitive mutants of Arabidopsis and tomato have implicated maternal tissue (fruit/testa) sensitivity to ethylene in the control of germination speed (Siriwityayawan et al., 2003).

Mutants with darker than usual testa appearance have been isolated in tomato on six different occasions (brownseed1, 2, 3, 4, lateral suppressor, blackseed; Soressi, 1967; Philouze, 1970; Yordanov and Stamova, 1971; Monti, 1972; Taylor, 1979; Downie et al., 2003b). In addition to bs1 and bs4 mutations’ negative impacts on seed germination percentage and rate, the BS5 (possibly BS6; Soressi, 1972) genes have been mapped to locations on chromosome one harbouring quantitative trait loci positively influencing salt-tolerant seed germination (Foord et al., 1998).

With the exception of the lateral suppressor mutation, the darker-than-usual testa mutants are all inherited as monogenic, recessive, Mendelian traits that are not determined exclusively by the maternal genotype, indicative of a paternal contribution in the control of testa attributes (Downie et al., 2003b). Mutation of the genes encoding factors participating in the paternal control of testa attributes disrupts communication between the endosperm and the testa and results in altered testa composition and/or aberrations in the identity of the endosperm cell file immediately beneath the testa (Downie et al., 2003b). This report elucidates bs physiological and genetic perturbations, relating them to the delayed germination phenotype.

Materials and methods

Plant material

Wild-type or mutant tomato (Lycopersicon esculentum Mill. cv. Moneymaker (MM and bs5) or Ailsa Craig (AC and bs5)) plants were grown, along with bs5, from seed in the University of Kentucky Horticulture Greenhouse complex (Buxton and Jia, 1999). Seeds were harvested, cleaned (0.1 M HCl for 1 h), washed in tap water, dried (5% moisture content on a fresh weight basis), and stored at −20 °C. The WT Pielalbo, the true background of the bs mutant, is determined exclusively by the maternal genotype, indicating that the darker-than-usual testa mutants are all inherited as monogenic, recessive, Mendelian traits that are not determined exclusively by the maternal genotype, indicative of a paternal contribution in the control of testa attributes (Downie et al., 2003b). Mutation of the genes encoding factors participating in the paternal control of testa attributes disrupts communication between the endosperm and the testa and results in altered testa composition and/or aberrations in the identity of the endosperm cell file immediately beneath the testa (Downie et al., 2003b). This report elucidates bs physiological and genetic perturbations, relating them to the delayed germination phenotype.

Mutant characterization

Seed weight, area, number per fruit, and fruit pH were determined as previously described (Downie et al., 2003b). Electron paramagnetic and puncture force-analysis, the ontology of dark pigment accumulation within the bs mutant seeds, epistasis analysis with the anthocyaninless mutants, enzyme assays, and reactive oxygen species measurements were performed as previously described (Downie et al., 2003b).

Seed germination

Eight replicates of 25 seeds each were plated onto three layers of germination blotter paper in square germination trays for each genotype. Four replicate blotters per genotype were saturated with distilled, deionized water and four with 100 μM GA4+7. The seeds were scanned every 12 h for 4 d using the Paradigm Seedlot Vigor Assessment™ system (Version 3.2; Paradigm Research Corp, South Haven, MN, USA) and mean percentage germination and mean germination time (MGT, Bewley and Black, 1994) for WT and mutant seed computed and compared using the Statistical Analysis Systems (1999; see below).

Seeds were harvested from fruit and fermented in the juice for 24 h to remove the sheath. The seeds were then apportioned into three equal fractions, and dried over activated alumina (Grabe, 1989) in an air-, nitrogen-, or oxygen-sparged desiccator. Following 3 d of dehydration, fresh seeds were harvested from fruit. Following 24 h fermentation they were cleaned and four replicates of 25 seeds each were picked immediately on water-saturated blotters as described above. At this point four replicates of 25 seeds each that had been dried for 4 d in each of the atmospheres were also plated on water-saturated germination blotters (see above). The undried control in this experiment was possible because tomato seeds do not require dehydration to complete germination (Berry and Bewley, 1991). Seeds were scanned every 12 h using the Paradigm System and mean percentage germination and mean germination time calculated from the data.

Molecular genetic assessment of differentially expressed genes between seeds of wild-type Moneymaker and bs5

Subtracted library construction: Mature seeds of Moneymaker and bs5 mutant plants were harvested and ground directly in RNA isolation buffer (Cooley et al., 1999). Poly A+ RNA was procured using Dynabeads (Dynal Biotech Inc., Lake Success, NY, USA). The mRNA was reverse transcribed and cDNA used to perform two subtractive, suppressive PCR (Diatchenko et al., 1996) experiments between Moneymaker and bs5 (forward and reverse selection) using a kit (PCR-select; BD Biosciences, Palo Alto, CA, USA). The resulting amplicons were size fractionated on a 1% (w/v) agarose gel and those fragments less than 500 bp, between 500 and 1000 bp, and greater than 1000 bp, were rescued from the gel. Size-fractionated amplicons were ligated into a homemade T/A cloning vector engineered to leave single, T' overhangs following XcmI cleavage (Q Xu and B Downie, unpublished data). Following transformation into DH5α, bacteria were titred on LB 100 μg ml−1 ampicillin. The libraries were diluted to −4 cfu μl−1, and 750 μl spread on solid LB media containing 100 μg ml−1 ampicillin, 1.6% w/v agar, 37.5 μM IPTG, and 0.0075% (w/v) X-GAL. Those colonies containing inserts were picked using blue/white screening and arrayed in 384 well plates (Q-Pix II, Genetix Ltd., New Milton, Hants, UK) containing LB 100 μg ml−1 ampicillin and 15% glycerol. Libraries were duplicated, bacteria grown to high density overnight using a HiGro II (Genomic Instrumentation Service, Inc. San Carlos, CA, USA) at 520 rpm, 8.3 ml of humid, ultrapure air introduced every 30 s, and frozen at −80 °C.

Filter preparation, hybridization and analysis: Libraries were retrieved from −80 °C, warmed to room temperature over several hours and arrayed on the bed of the Q-Pix II (Genetix) fitted with a 384 pin arraying head. A 492 cm2 piece of HyBond N+ membrane (Amersham-Pharmacia, Piscataway, NJ, USA) was placed on an LB 100 μg ml−1 ampicillin-soaked 3MM filter paper (Whatman, Maidstone, Kent, UK) on the gridding block and bacteria transferred
from the library and arrayed in a 4 × 4 G duplicate pattern as microdots on the membrane according to the manufacturer’s instructions (Genetix). The membrane was removed from the filter paper and placed on a Q-Trap (Genetix) containing 200 ml of LB 100 μg ml −1 ampicillin, 1.6% (w/v) agar. An LB 100 μg ml −1 ampicillin-soaked 3MM filter paper (Whatman) was placed in the lid to provide a moist atmosphere and the whole inverted and incubated for 20 h at 37 °C to permit colony growth. The membrane was removed from the LB, placed on 3MM filter paper soaked with denaturation solution (0.5 M NaOH, and 1.5 M NaCl) over a boiling water bath for 4 min. The filter was removed to a 3MM filter paper soaked in neutralization solution (1 M TRIS-HCl, pH 7.5, 1.5 M NaCl) for 4 min and then dried on 3MM filter paper for 1 min. Next the membrane was inverted (colony side down) in 100 ml, temperature pre-equilibrated proteinase K solution (0.1 mg ml −1 proteinase K [F-Hoffman-La Roche, Basel, Switzerland], 50 mM TRIS-HCl, pH 8.0, 1% w/v sarkosyl, 100 mM NaCl, 50 mM EDTA) at 37 °C for 1 h. The membrane was removed from the proteinase K solution, placed on a 3MM filter paper and a second filter paper placed on top of the membrane was removed from the proteinase K solution, placed on a 3MM filter paper and a second filter paper placed on top of the membrane. A pipette was used to roll the filter paper onto the membrane and the whole was left to dry overnight. The following day, the top filter paper was peeled from the membrane and the dry membrane UV-crosslinked at 50 mJ (GS GENE LINKER, Bio-Rad Laboratories, Hercules, CA, USA).

**Probe preparation, hybridization conditions, image analysis:** The probe for colony macro-arrays and northern blot was prepared using PCR and adaptor primers in the presence of [32P]-dATP (New England Nuclear, Boston, MA, USA). The probe for macro-arrays was synthesized en masse from subtracted or un-subtracted cDNAs according to the kit manufacturer (BD Biosciences). Probes for northern blot used plasmid prepared from single colonies previously identified as being differentially expressed between the two populations by macro-array. Following PCR, the radiolabelled cDNAs for the macro-arrays were cleaved with RsaI to remove the adaptors (also present on the ampiclons constituting the library). Thereafter, the probes for either the macro-arrays or the northern blot were run through a PCR purification column (Qiagudix, Qiagen, Valencia, CA), washed, and eluted in 50 μl. Aliquots were counted using scintillation, the probes boiled, quenched, and added to the hybridization solution.

Macro-array or northern blot membranes were blocked at 68 °C for 12 h in pre-hybridization solution (6× SSPE, 5× Denhardt’s solution (Denhardt, 1966), 0.5% SDS, and 100 μg ml −1 boiled, sheared salmon sperm DNA). Thereafter, the membranes were hybridized with (for macro-arrays) equal counts of boiled, quenched probe introduced into fresh pre-hybridization solution. Following hybridization, membranes were first washed twice, 15 min each time, at low stringency (2× SSC, 0.1% SDS at 68 °C) and exposed to a phosphor screen for 2 d. The image was captured using a PhosphorImager 445 SI (Molecular Dynamics, Sunnyvale, CA, USA) and the blots re-washed at high stringency (0.2× SSC, 0.1% SDS at 68 °C for 30 min) and re-exposed to the phosphor screen for 5 d.

Colonies harbouring ESTs significantly up- or down-regulated between the two genotypes were identified from images produced from the PhosphorImager and, for some, plasmid DNA was isolated for use in northern blot and sequencing.

**Sequencing:** Cycle sequencing reactions employed universal primers (Integrated DNA Technologies, Inc., Coralville, IA, USA) binding to the plasmid oriented toward the T/A cloning site and DTCS chemistry (Beckman Coulter, Inc., Fullerton, CA, USA) run on a GeneAmp 9700 PCR machine (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were cleaned using magnetic bead technology (Agencourt Bioscience Corp., Beverly, MA, USA) and sequencing was performed at the Advanced Genetics Technologies Center (AGTC) (University of Kentucky, Lexington, KY, USA) using a Beckman Coulter CEQ 8000XL, eight capillary electrophoresis Genetic Analysis System. The proprietary UK-AGTC LIMS system was employed to perform automated sequence quality analysis, vector/adaptor masking, and database homology searches.

**Statistical analysis**

All comparisons of the effect of the mutations were performed within a cultivar (AC with bs4; MM with bs4). Seed weight, number of seed per fruit, fruit pH, enzyme activity, increases in moisture content during imbibition, and ROS release into the media were subjected to analysis of variance using the ANOVA procedure of Statistical Analysis Systems (1999). The analysis of the force necessary to puncture the micropylar tip, final percentage germination, and MGT was performed between genotypes (mutant versus WT) within a drying treatment. Because it was not possible to obtain the Pieralbo WT background of bs4, the mutant was left out of this analysis. The effect of drying treatment on puncture force, final percentage germination, and MGT were compared within each genotype (this included bs4). If the ANOVA indicated that there were significant differences among means, Tukey’s mean separation test was used to distinguish among them.

**Results**

**Testa toughness, germination percentage, and speed**

The completion of germination of the afterripened, darker-than-usual testa mutants was significantly delayed relative to the afterripened seeds of the respective WTs except for bs4 and WT AC imbibed on water (Fig. 1A, B). The final germination percentage of bs4 and WT AC were not significantly different with or without GA4 + 7 (Fig. 1A, B). This was not the case for bs4 mutant seeds, although the percentage germination of these seeds after 10 d on GA4 + 7 was double that on water it was still significantly less than WT MM (Fig. 1A, B). The delay in completion of germination of the darker-than-usual testa mutants, when it occurred, was not due to a deficiency in imbibition rate (Fig. 2).

Previous work suggested a requirement for seed dehydration to enhance testa toughness in a blackseed mutant relative to the wild type (Downie et al., 2003). To test this characteristic in the bs mutants, germination tests and puncture force analyses 24 HAI were conducted on freshly harvested mutant and respective WT seeds that had been: (1) harvested and dried in air; (2) harvested and placed directly on water without drying (fresh); (3) harvested and dried in N2; and (4) harvested and dried in O2.

Comparison of bs4 mutant seed with WT AC dried under a variety of conditions supported the contention that oxygen exacerbated testa toughness differences (Table 1). Dehydration in nitrogen eliminated differences in testa toughness between the two genotypes (Table 1). However, greater bs4 testa toughness did not necessarily result in a greater MGT or poorer final germination percentage (Table 1).
The presence of oxygen during dehydration did not result in greater testa toughness for $bs^4$ (Table 1). However, $bs^4$ seeds dried in oxygen completed germination significantly poorer than did WT MM seeds dried in oxygen. This was also the case for seeds germinated fresh after cleaning (Table 1), while seeds dried in air or nitrogen completed germination to the same extent as WT MM (Table 1). It was concluded that drying $bs^4$ seeds in the presence of oxygen was no more detrimental than drying WT MM seeds in the same atmosphere.

Comparing air-dried seeds with fresh seeds, there was no consistent trend in germination performance. Placing WT seeds directly on water did not affect or significantly decreased mean germination time, was deleterious or inconsequential to the final percentage germination, and did not affect the puncture force attained by WT AC and WT MM, respectively (Table 1). Drying under any atmosphere exacerbated the deleterious effect of the dark testa colouration on the final percentage germination for $bs^2$, while air-dried $bs^4$ seeds completed germination to a greater percentage than $O_2$-dried or fresh seeds (Table 1). Fresh seeds from $bs^1$ plants attained a faster MGT than did air- or $N_2$-dried seeds while most fresh $bs^4$ seeds failed to complete germination (Table 1).

Treatments tending to produce low puncture forces also tended to have faster MGTs for all genotypes except $bs^1$ (Table 1). The converse relationship was also true for all genotypes except $bs^1$ where tougher seeds had slower MGTs (Table 1). In addition, except for $bs^2$, treatments with slower MGTs and tougher seeds also completed germination poorer (Table 1).

**General characterization**

A description of the ontogeny of pigment accumulation and localization in the $bs$ mutants can be found in Downie et al. (2003b).

The seeds of the $bs$ mutants were all heavier and larger than seeds from their respective WTs (Table 2). The number of seeds per fruit was unaffected by the mutations.
The physical toughness of the testa (force in Newtons) was also influenced by the imposition and manner of dehydration. Fresh, not dried prior to germination on water; Air, N₂, O₂, dried under an air, nitrogen, or oxygen atmosphere, respectively, over activated alumina for 4 d prior to germination on water. Lower case letters following the standard error of the mean denote significant differences within a cultivar (WT AC versus bs¹; WT MM versus bs⁴). The WT Pieralbo, the bs² background, is unavailable for comparison. (Table 2). No discernible difference in fruit pH was determined for bs¹ relative to WT AC.

Ailsa Craig seeds, both WT and bs¹, contained unpaired electrons, as determined using EPR (Table 3). The peak was at the same frequency (3475 G) as that for blackseed (bks) mutant tomato seeds (Downie et al., 2003b), black sunflower (Helianthus annuus L.), and niger (Guizotia abyssinica (L.S.) Cass.). The latter two species and the recently characterized mutant tomato are known to produce seeds with testae comprised of melanic compounds. However, unlike the bks seeds (Downie et al., 2003b), hyperproduction of a melanic compound does not appear to be the chemical alteration of the bs mutants resulting in aberrations in seed germination because both wild-type Ailsa Craig and bs¹ seeds had similar EPR spectra. Neither wild-type Moneymaker nor bs⁴ exhibited appreciable EPR signals, which is attributed to a lack of unpaired electrons, a hallmark of melanic compounds (Table 3).

**Table 1. The final germination percentage and mean germination time (MGT) of wild-type Ailsa Craig, Moneymaker, bs¹, bs², and bs⁴ mutant seeds was influenced by how the seeds were dried**

The hypothesis was therefore tested that oxygen or a ROS was interacting with some testa component in bs¹, bs², and bs⁴, to crosslink and darken (in some instances, toughen) the testa. Toughening could be enzymatically mediated by either peroxidase (PRX) or catalase-insensitive PPO activity among the genotypes (data not shown). However, the bs⁴ mutants exhibited considerable PRX activity relative to WT (Fig. 3A, B). Incubation activity staining revealed that the bs¹ mutant seed also had detectable PRX activity while WT AC did not, but activity assays were unable to document a difference.

**Table 2. Seed weight, seed planar area, seed number per fruit, and fruit pH of mutant and respective WTs**

Lower case letters following the standard error of the mean denote significant differences within a cultivar (WT AC versus bs¹; WT MM versus bs⁴). The WT Pieralbo, the bs² background, is unavailable for comparison.

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**Reactive oxygen species (ROS) scavenging enzymes were up-regulated in bs testa mutants**

Shikimate compounds accumulate in testae and may be activated by peroxidase-mediated reduction of peroxide into oxidative polymerization producing compounds that increase testa strength (Gillikam and Graham, 1991; D’Ischia et al., 1991; Todd and Vodkin, 1993; Gijzen, 1997). Previous work has demonstrated an up-regulation of peroxidase in bks mutant seeds relative to WT (Downie et al., 2003b). The hypothesis was therefore tested that oxygen or a ROS was interacting with some testa component in bs¹, bs², and bs⁴ to crosslink and darken (in some instances, toughen) the testa. Toughening could be enzymatically mediated by either peroxidase (PRX) or polyphenol oxidase (PPO). There was no difference in catalase-insensitive PPO activity among the genotypes (data not shown). However, the bs⁴ mutants exhibited considerable PRX activity relative to WT (Fig. 3A, B). Incubation activity staining revealed that the bs¹ mutant seed also had detectable PRX activity while WT AC did not, but activity assays were unable to document a difference.
Many ESTs were differentially expressed between WT MM and bs4 seeds

Seeds of the bs4 mutants were considerably delayed in radicle protrusion and did not attain the percentage germination of WT MM seeds (Fig. 1; Table 3). Furthermore, bs4 mutant seeds were perturbed in aspects of ROS scavenging activities (Fig. 3) and mis-identified the outermost cell wall of the endosperm as testa (Downie et al., 2003b). These aberrations suggested that there might be considerable differences in the transcriptional activity between bs4 and WT MM seeds.

When plasmid cDNA libraries of size-fractionated, subtractive, suppression PCR amplicons (Fig. 6A) were arrayed and identical filters challenged with cDNA probes of radiolabelled subtracted and unsubtracted libraries, many putatively differentially expressed cDNAs between the two genotypes were identified (Fig. 6B, C). Northern blot analysis of poly A+ RNA from developing seeds from maturing fruit staged as ‘pink’ (Fig. 6D) or total RNA from mature, quiescent or 24 HAI bs4 and WT MM seeds (Fig. 7), independently verified that some of the genes tested were differentially expressed between the two genotypes at some stage. Those ESTs up-regulated in bs4 relative to WT MM and identifiable with orthologues in public repositories, were associated with seed development (Fig. 7).

Discussion

Pleiotropic effects of the bs mutants

The fruit from the bs4 mutants had greater than usual pH, a phenotype reported for fruit from bs1 mutants (Martiniello et al., 1985), but not observed in this study. Many phenolic acids (shikimate metabolites) accumulate in tomato fruit tissue as maturation progresses (Walker, 1962; Buta and Spaulding, 1997). Disruption of the normal accumulation of one or more of these compounds in the fruit could alter fruit pH. The resultant accumulation of this precursor or alternative shikimate compound in the testa could participate in darkening the testa more extensively than usual.

Seed germination

The poor germination rate and percentage (bs4) of bs seeds relative to smaller, lighter AC and MM WT seeds is consistent with previously published conclusions (Whittington and Fierlinger, 1972) that, for tomato, small seed size leads to faster completion of germination. However, Atanassova et al. (1997a) and Downie et al. (2003b) could not find a consistent relationship between seed weight and rate of germination for anthocyaninless or blackseed tomato mutants, respectively. Afterripened bs1 mutant seeds attained a final percentage germination comparable with WT AC on both water and 100 µM GA4+7 (Fig. 1A, B). These results argue against the poor

<table>
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*a* NAS indicates no appreciable signal.

(Fig. 3A, B). Catalase (CAT) activity was statistically significantly greater (α=0.05) in bs1 and bs4 mutant seeds relative to WT AC and WT MM, respectively (Fig. 3C). In-gel staining for CAT activity following native PAGE did not reveal obvious differences in CAT (Fig. 3D). There were no significant differences in superoxide dismutase (SOD) activity among the genotypes (Fig. 3E). Using SDS-PAGE, without boiling the sample in SDS-loading buffer, it was determined that similar SOD isoforms were present in the seeds of all genotypes (Fig. 3F).

Statistically significantly greater amounts (α=0.05) of reactive oxygen species (ROS) were released from intact, dry WT MM seeds than from those of bs4 (Fig. 4). This difference was substantially mitigated 24 HAI, but WT MM seeds still released statistically significant greater amounts (α=0.05) of ROS into the media than bs4 early during the test (Fig. 4). No such statistical difference was apparent, regardless of imbibition status of the seed, for bs1 and its wild type.

bs testa mutants were epistatic to anthocyaninless mutants

Testa attributes of the tomato anthocyaninless mutants anthocyaninless of Hoffman (ah), without anthocyanin (aw), and baby lea syndrome (bls) include: (1) a lighter than WT testa colour; (2) a pattern of inheritance dependent on the genotype of the testa (Atanassova et al., 1997a); and (3) more rapid completion of germination than WT (Atanassova et al., 1997a, b). These mutants along with anthocyaninless (a), anthocyanin absent (aa), entirely anthocyaninless (ae), anthocyanin free (af), anthocyanin gainer (ag), anthocyanin gainer-2 (ag-2), anthocyanin loser (al), and anthocyanin reduced (are) were all tested for epistasis with the bs mutants. Double mutant plants (seedlings with green hypocotyls from bs seeds) were produced (except for bs2×are, despite several attempts) and F3 seed collected and compared with seeds from WT and bs plants. In every case examined, the bs mutants were epistatic to the anthocyaninless mutants (Fig. 5).
germination of this mutant being due to the effects of the tests on light quality. The dark pigment in the mutant tests was also unlikely to be a germination inhibitor (Walker, 1962) because, when the tests and endosperm cap opposing the radicle were removed from $bs^2$ mutant seeds, the radicle protruded readily and most established as seedlings (data not shown). Finally, although dark testa coloration has frequently been associated with inhibition of water uptake in seeds during imbibition (Egley et al., 1983) this is not the cause of delayed germination in the $bs$ mutants (Fig. 2). Based on puncture force analysis, one of the reasons the $bs^4$ mutant seed is delayed in the completion of germination is due to a greater testa mechanical restraint (Table 1). This is not, however, the case for the $bs^4$ mutant seeds which had a mechanical restraint equal to or less than that of all but fresh WT MM seeds (Table 1).

Fig. 3. The activity of (A) peroxidase (PRX), (C) catalase, and (E) superoxide dismutase was assayed in mutant and wild-type seeds (the $bs^2$ parental line, Peralbo is unavailable for comparison). Different lowercase letters in or above the bars depicting enzyme activity denote statistically significant ($\alpha=0.05$) activities between genotypes within a cultivar (e.g. WT MM and $bs^4$). (B) Native PAGE (12% w/v, 20 μg buffer-soluble protein lane$^{-1}$) stained for PRX activity from WT and mutant seeds. PRX represents a commercial preparation. (D) A native PAGE gel (10 μg buffer soluble protein lane$^{-1}$) of CATALASE activity. (F) SDS-PAGE of crude protein extracts (20 μg lane$^{-1}$) from WT and mutant seed dissolved in SDS-loading buffer but not boiled, stained for superoxide dismutase (SOD) activity revealed that the identity of SOD isoforms was invariant between the WT and mutant seeds. SOD represents a commercial preparation. kD: kiloDaltons.
Many seeds of both WT MM and bs4, when harvested fresh, were dormant, resulting in low percentage germination even for the WT (Table 1). Dormancy is a trait of greenhouse-grown MM seeds that has been observed previously (Downie et al., 2003a). Afterripening WT MM seeds at 25 °C for 4 months prior to imbibition on water alleviated dormancy and allowed 100% completion of germination. The final germination percentage of bs4 seeds on water was also marginally improved by afterripening and a combination of afterripening and imbibition on GA4+7 greatly improved bs4 germination percentage (Fig. 1B).

Reactive oxygen species scavenging enzymes were up-regulated in the bs mutants

It is difficult to determine whether the dark testa colour of the mutants is associated with greater ROS scavenging enzyme activity or if the two are divorced consequences of a pleiotropic mutation. Enzymes associated with ROS scavenging have been implicated previously with developmental processes in the testa and/or seed germination processes (Gross, 1977; Cassab and Varner, 1987; McClung, 1997; Downie et al., 2003b). One report has suggested that inhibition of CAT is required for dormancy alleviation by redirecting H2O2 to participate in the oxidation of NADPH thus permitting the pentose phosphate pathway to proceed (Hendricks and Taylorson, 1975). In maize, constitutive CAT activity during seed development can be stimulated, and during germination, inhibited, by the flavonoid-derived phytohormone, salicylic acid (Guan and Scandalios, 1995). However, there is no evidence of CAT affecting the testa directly while NADPH availability rather than NADP amounts appear limiting for successful dormancy alleviation (Lozano et al., 1996).

It is possible that ROS generation is greater in the bs1 and/or bs4 mutants, but that, in the first instance, the up-regulation of CAT may decrease the steady-state amounts of ROS to WT levels while in the second, up-regulation of both PRX and CAT decreases ROS amounts below that of WT MM. Conversely, ROS generation may be normal in mutant seed and the up-regulation of CAT or PRX activity may be a peripheral consequence of the lesion, divorced from ROS generation.

Fig. 4. The amount of reactive oxygen species released from mutant and wild-type seeds during or 24 h after imbibition was analysed. Different lower case letters for each time point between the genotypes signify statistically significantly different mean ROS release. There were no significant differences between bs1 and WT AC in ROS release at any time point and so these curves were not lettered.
The epistatic relationship between bks and anthocyaninless mutants

Precedence exists for the colour of seed coats (testa, pericarp) to be imparted by flavonoid metabolites (Mol et al., 1998). Reports in the literature of tomato mutants affected in anthocyanin production producing seeds with lighter than usual testa colour (ah, aw, bls; Atanassova et al., 1997a) led to the speculation that the flavonoid biosynthetic pathway was functioning to produce condensed tannins in the tomato testa in much the same way it does in Arabidopsis.

The dark testa colour of the double mutants was in no case even mitigated, let alone eliminated, when combined with the anthocyaninless mutants. Hence, the anthocyaninless mutants are not epistatic to the bs mutants. However, for reasons developed previously (Downie et al., 2003b) it is not possible to determine if the bs mutants are truly epistatic to the anthocyaninless mutants or simply masking the lighter anthocyaninless testa colour.

Contrasting patterns of inheritance between anthocyaninless and the bs mutants

The manifestation in the seed of all reported tt, ats, mum (Arabidopsis), and anthocyaninless (tomato) mutants affecting the biochemical and physical properties of the testa depends solely on the genotype of the maternal parent (i.e. the genetic composition of the maternally derived testa; Atanassova et al., 1997a). However, the bs1,2,4 and bks mutants are all inherited as recessive, monogenic, Mendelian traits (i.e. their phenotype is not determined strictly by the genotype of the testa). There appears to be antagonism between the maternal testa and the endosperm/embryo with their paternal genetic contribution that influences testa colour and, in some instances, toughness (Ellner, 1986). This necessitates communication between the testa and the underlying endosperm/embryo with the latter mitigating the full potential of the testa to darken and, in some instances, toughen (Downie et al., 2003b).

Differences among bs/bks mutants

Although manifestations of the bs/bks mutations are similar (slow completion of germination, dark testa colour, inheritance independent of the testa genotype), differences are also evident. All the bs seeds were larger and heavier than their respective WT while bks mutant seeds are smaller and lighter (Downie et al., 2003b). The bs4, but not the other mutant seeds, accumulate pigment in the periclinal, secondarily thickened cell walls of the first endosperm cell file (Downie et al., 2003b), and increased germination percentage in response to 100 μM GA4+7. The force required to penetrate the micropylar endosperm and testa was significantly greater for air-dried bs and bks seeds 24 HAI on water relative to their respective WTs.
However, such was not the case for air-dried \( bs^4 \) seeds (Table 1). There was no consistent effect of the atmosphere under which the \( bs \) seeds were dried on germination rate, final germination percentage, or testa toughness. This contrasts with \( bks \) mutant seeds for which the germination percentage and speed increased, and the puncture force decreased, when drying in air was avoided (Downie et al., 2003b). The different lesions varied in the degree of severity of their effect on final germination percentage and speed despite a similar testa appearance. Different combinations of ROS scavenging enzymes were significantly up- or down-regulated in the different mutants relative to their respective WT\( s \) (Fig. 3) and \( bks \) mutant seeds accumulate free radicals while WT MT did not. In conclusion, the \( bs^1 \), \( bs^3 \), and \( bks \) mutants do not represent aberrations that are manifest through the same biochemical/physiological processes.

**Perturbations in the transcriptome of \( bs^4 \) seeds: failing to exit late development?**

The wide range of ESTs recovered from the subtractive, suppressing PCR libraries and their differential expression between the two genotypes, confirmed hypotheses that the

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**Fig. 6.** Differential expression between WT MM and \( bs^4 \). (A) Subtractive, suppressive PCR produced two libraries enriched for transcripts preferentially expressed in WT MM or \( bs^4 \) seeds. The degree to which the libraries were enriched for differentially expressed transcripts was tested using tubulin-specific primers on aliquots of the subtracted and unsubtracted libraries. (B) Subtracted libraries were arrayed in duplicate on identical HyBond N\( ^+ \) membranes, and separate membranes were probed with labeled cDNAs made from the unsubtracted MM library. (C) Arrays were probed with labeled cDNAs made from each unsubtracted and subtracted library. Images were compared and differentially regulated clones identified. Some of the differentially regulated clones were picked, amplified, and sequenced. (D) Select clones were used as probes on Poly A\( ^+ \) (from developing seeds from maturing fruit staged as 'pink') northern blots to verify differential expression during late development.
bs^4 mutation represents a pleiotropic diversion from the normal developmental program suggested by: (1) the mutation’s effects on seed attributes (size, weight, colour); (2) differences in ROS scavenging enzyme activity; and (3) fruit attributes.

Only one of the differentially expressed transcripts identified as up-regulated in bs^4 mutant seed was retrieved from a differential cDNA display (DCD) analysis of gibberellic acid deficient (gib-1) seeds imbibed on water or 100 µM GA_4+7 for 40 h (Cooley et al., 1999; KJ Bradford, personal communication). The LeSNF4, the regulatory subunit of the sucrose non-fermenting protein kinase subfamily, was up-regulated in gib-1 seeds on water relative to these seeds on GA_4+7 (Bradford et al., 2003) and is up-regulated in bs^4 mutants seeds relative to WT MM (data not shown). Other than LeSNF4, the paucity of identical cDNAs between the two analyses might be because the DCD analysis examined seeds during germination while the current study examined seeds that had completed development. Although, in tomato, the two processes of development and germination can be closely associated in time, with no requirement for maturation desiccation and the incidence of precocious germination in the fruit fairly common, the corresponding change in gene expression pattern is thought to be profound (Kermode, 1990; Ogas et al., 1999). In addition, while gib-1 seeds are prevented from timely completion of germination, relative to WT or gib-1 with exogenous GA_4+7, by mechanical restraint (Groot and Karssen 1987), bs^4 seeds are probably not. The gibberellic acid biosynthetic pathway is intact in bs^4 mutants producing plants of normal stature, and the testa was not significantly tougher than that of WT MM seeds.

To date, no clones encoding a PRX or CAT have been retrieved from the bs^4-enriched library. However, sequence data for clones from the bs^4 library subtracted by MM (i.e. present in greater abundance in bs^4) have identified ESTs (including LeSNF4, Bradford et al., 2003) whose products are associated with late seed development rather than germination (Table 1). The greater expression in quiescent and 24 HAI bs^4 relative to WT MM seeds of genes encoding an oleosin (Aalen et al., 1994), storage protein (Bewley and Black, 1994), and a dehydrin (Han et al., 1997) suggest that the bs^4 mutant continues in the developmental programme following maturation desiccation. A substantial CAT activity, associated with seed development (Suzuki et al., 1995) and normally transiently repressed during germination (McClung, 1997) supports this contention as does the response of the bs^4 seeds to GA_4+7, a hormone known to stimulate the germinative program (Cooley et al., 1999; Bradford et al., 2003). A failure to exit the late developmental programme may also explain why the bs^4 mutant continues in the developmental programme following maturation desiccation. A substantial CAT activity, associated with seed development (Suzuki et al., 1995) and normally transiently repressed during germination (McClung, 1997) supports this contention as does the response of the bs^4 seeds to GA_4+7, a hormone known to stimulate the germinative program (Cooley et al., 1999; Bradford et al., 2003). A failure to exit the late developmental programme may also explain why the bs^4 seeds are larger and heavier (a longer duration/more intense period of seed filling). The darker testa colour in bs^4 mutant seeds may be due to the prolonged production of testa precursors. Once the testa dies, these precursors may be trapped in the endosperm and polymerize in the outermost periclinal wall (Downie et al., 2003b). These suppositions are all harmonious with the recessive, Mendelian inheritance of the bs^4

<table>
<thead>
<tr>
<th>Accession</th>
<th>Best homologue in the databases</th>
<th>E-value</th>
<th>Library</th>
<th>Northern lanes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF24307</td>
<td>Y08427 N. tabacum/b. internal transcribed spacer between 26S and 18S rRNA genes</td>
<td>2e^-12</td>
<td>bs^4</td>
<td>1 2 3 4</td>
</tr>
<tr>
<td>CF24308</td>
<td>AAK63331 Lyococcus escuientum accession lys21 gene for 25 seed albumin-1 large subunit</td>
<td>6e^-70</td>
<td>bs^4</td>
<td>+</td>
</tr>
<tr>
<td>CF24309</td>
<td>No match</td>
<td></td>
<td>WT MM</td>
<td>+</td>
</tr>
<tr>
<td>CF24400</td>
<td>No match</td>
<td></td>
<td>bs^4</td>
<td>-</td>
</tr>
<tr>
<td>CF24401</td>
<td>AY073291 Arabidopsis thaliana unknown protein (AK204770) mRNA</td>
<td>3e^-27</td>
<td>bs^4</td>
<td>+</td>
</tr>
<tr>
<td>CF24402</td>
<td>A049862565 Quercus robur mRNA for geranyl dipiphosphate synthase (gpd gene)</td>
<td>1e^-10</td>
<td>WT MM</td>
<td>+</td>
</tr>
<tr>
<td>CF24403</td>
<td>Arabidopsis thaliana 26S proteasome regulatory subunit (RP11:NM_122261) similar to 26S proteasome regulatory particle non-ATPase subunit 11 (Os11G17297G) from Oryza sativa</td>
<td>5e^-60</td>
<td>WT MM</td>
<td></td>
</tr>
<tr>
<td>CF24404</td>
<td>AY156239 Lyococcus escuientum 17.8 kDa class I small heat shock protein (KSP17.8) mRNA</td>
<td>6e^-10</td>
<td>WT MM</td>
<td>+</td>
</tr>
<tr>
<td>CF24405</td>
<td>V15613 Solanum commersonii mRNAs for DHN1 protein, a dehydrin-like gene</td>
<td>3e^-39</td>
<td>bs^4</td>
<td>+</td>
</tr>
<tr>
<td>CF24406</td>
<td>AK103333 Oryza sativa (zona indica cultivar group) mRNA cDNA clone: J033129008</td>
<td>2e^-29</td>
<td>bs^4</td>
<td>+</td>
</tr>
<tr>
<td>CF24407</td>
<td>No match</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CF24408</td>
<td>Zea mays FCO062072 mRNAs sequence, AK117715 Arabidopsis thaliana Atg1670 mRNA for unknown protein</td>
<td>5e^-10</td>
<td>WM MM</td>
<td>+</td>
</tr>
<tr>
<td>CF24409</td>
<td>U07793 Sesamum indicum 15.5 kDa oleosin mRNA</td>
<td>7e^-71</td>
<td>bs^4</td>
<td>+</td>
</tr>
<tr>
<td>CF358967</td>
<td>No match</td>
<td></td>
<td>WM MM</td>
<td>-</td>
</tr>
<tr>
<td>CF358968</td>
<td>S. subcrenatum mRNA encoding homologous to human P23 tumor protein</td>
<td>5e^-39</td>
<td>bs^4</td>
<td>+</td>
</tr>
</tbody>
</table>

Fig. 7. Summary of sequence analysis of representative, differentially expressed cDNAs between bs^4 and WT MM. Each EST was assigned an accession, and its homology (if any) to known clones in public repositories and E-value for that homology determined. The library from which the EST was retrieved was tracked. Additionally, expression of each EST in mature dry seeds (lanes 1 and 2) or 24 h-imibed seeds (lanes 3 and 4) of WT MM (lanes 1 and 3) and bs^4 (lanes 2 and 4) was analysed with northern blots of total RNA.
lesion. Whether the other bs mutants or the bks mutant also fail to exit the developmental programme is under investigation.

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