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RESEARCH PAPER

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

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## Abstract

**The *brownseed* mutants (*bs*[1], *bs*[2], *and bs*[4]) of tomato all possess dark testae and deleteriously affect seed germination speed and/or final percentage. Poor germination performance of the *bs*[1] but not the *bs*[4] mutant, was due to greater impediment to radicle egress. Testa toughening (*bs*[1]) was prevented by drying in $N_2$. However, poor germination speed was hardly affected by drying. $GA_{4+7}$ did not ameliorate germination percentage or speed (*bs*[1], *bs*[2]), whereas *bs*[4] seeds commenced radicle protrusion sooner and had a greater germination percentage. *bs*[1] mutant seeds have two times more catalase activity while those of *bs*[4] contained six times more peroxidase and almost two times more catalase activity than WTs. *bs*[4] release only half of the reactive oxygen species into the media than WT during imbibition. EPR detected the presence of free radicals in *bs*[1] 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 *bs*[4] and WT. EST identity suggests *bs*[4] does not exit the developmental programme upon attaining maturity.**

Key words: Catalase, electron paramagnetic resonance (EPR), free radicals, germination, *Lycopersicon 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*., 1997*a*, *b*; 2001; Debeaujon and Koornneef, 2000; Downie *et al*., 2003*b*) 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*., 1997*a*). 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-

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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 (Siriwitayawan *et al*., 2003).

Mutants with darker than usual testa appearance have been isolated in tomato on six different occasions (*brownseed*$^{1, 2, 3, 4}$, *lateral suppressor*, *blackseed*; Soressi, 1967; Philouze, 1970; Yordanov and Stamova, 1971; Monti, 1972; Taylor, 1979; Downie *et al*., 2003*b*). In addition to *bs*$^1$ and *bs*$^4$ mutations' negative impacts on seed germination percentage and rate, the *BS*$^1$ (possibly *BS*$^4$; Soressi, 1972) genes have been mapped to locations on chromosome one harbouring quantitative trait loci positively influencing salt-tolerant seed germination (Foolad *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*., 2003*b*). 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*., 2003*b*). 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 *bs*$^4$) or Ailsa Craig (AC and *bs*$^1$)) plants were grown, along with *bs*$^2$, 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 Pieralbo, the true background of the *bs*$^2$ mutant, is unavailable. The *bs*$^2$ mutant was included in this report to document its germination habit and physical attributes. Due to similarities in germination behaviour, *bs*$^2$ seed attributes are reported in the figures and tables with those of *bs*$^1$/wild-type Ailsa Craig (WT AC).

### Mutant characterization

Seed weight, area, number per fruit, and fruit pH were determined as previously described (Downie *et al*., 2003*b*). Electron paramagnetic- and puncture force-analysis, the ontogeny 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*., 2003*b*).

### 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 $GA_{4+7}$. The seeds were scanned every 12 h for 14 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 sown 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 bs$^4$

*Subtracted library construction*: Mature seeds of Moneymaker and *bs*$^4$ 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 *bs*$^4$ (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 *Xcm*I 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 $\mu l^{-1}$, 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 $cm^2$ 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 micro-dots on the membrane according to the manufacturer's instructions (Genetix). The membrane was removed from the filter paper and placed on a Q-Tray (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 denaturing 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. 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 [$^{32}$P]-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 *Rsa*I to remove the adaptors (also present on the amplicons constituting the library). Thereafter, the probes for either the macro-arrays or the northern blot were run through a PCR purification column (Qiaquick, 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 $bs^1$; MM with $bs^4$). 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 $bs^2$, 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 $bs^2$). 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 $bs^1$ and WT AC imbibed on water (Fig. 1A, B). The final germination percentage of $bs^1$ and WT AC were not significantly different with or without $GA_{4+7}$ (Fig. 1A, B). This was not the case for $bs^4$ mutant seeds, although the percentage germination of these seeds after 10 d on $GA_{4+7}$ 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*b*). 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 $N_2$; and (4) harvested and dried in $O_2$.

Comparison of $bs^1$ 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 $bs^1$ testa toughness did not necessarily result in a greater MGT or poorer final germination percentage (Table 1).

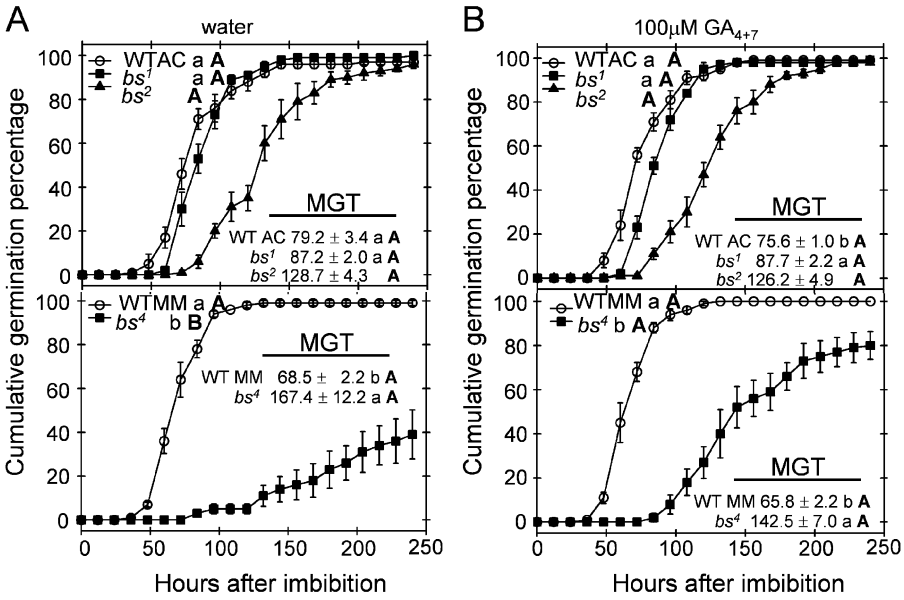



**Fig. 1.** Germination time-courses for mutant and wild-type seed on water or 100 μM $GA_{4+7}$. Mean germination time (MGT) ± standard error is presented for each genotype. The symbols for each genotype and the MGT standard error are followed by letters depicting significant differences. Different lower case letters denote statistically significant differences (α=0.05) between genotypes within a treatment (e.g. WT AC versus *bs*$^1$ on GA). Different upper case, bold letters denote significant differences (α=0.05) between treatments within a genotype (water versus GA for *bs*$^1$).

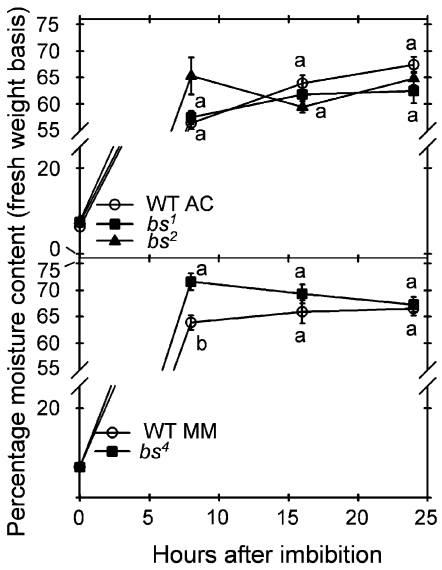



**Fig. 2.** Mutant and wild-type seeds were imbibed on water at 25 °C for 0, 8, 16, and 24 h and their moisture content determined. Different lower case letters denote statistically significant differences (α=0.05) between genotypes.

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*. (2003*b*).

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

**Table 1.** *The final germination percentage and mean germination time (MGT) of wild-type Ailsa Craig, Moneymaker,* bs[1], bs[2], *and* bs[4] *mutant seeds was influenced by how the seeds were dried*

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, $N_2$, $O_2$, 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 between genotypes within a drying treatment and cultivar. Upper case letters denote significant differences among drying treatments within a genotype. Wild-type Pieralbo, the parental line of *bs*[2], was unavailable for comparison to the mutant.

| Genotype | | $N_2$ | $O_2$ | Air | Fresh |
|---|---|---|---|---|---|
| WT AC | % Germination | 99.0±1.0 a A | 97.0±1.0 a A | 94.0±2.6 a A | 72.0±4.3 b B |
| | MGT (h) | 81.6±1.3 b A | 91.7±1.2 b A | 132.1±39.8 a A | 160.8±30.5 a A |
| | Puncture force (N) | 0.86±0.02 a A | 0.63±0.04 b B | 0.79±0.03 b A | 0.89±0.01 b A |
| *bs*[1] | % Germination | 91.0±2.5 b B | 96.0±1.6 a AB | 94.0±1.2 a AB | 100.0±0 a A |
| | MGT (h) | 136.7±5.9 a AB | 124.5±6.2 a BC | 148.2±1.4 a A | 112.9±4.5 a C |
| | Puncture force (N) | 0.86±0.02 a C | 1.06±0.05 a A | 0.94±0.02 a BC | 0.97±0.02 a AB |
| *bs*[2] | % Germination | 57.0±5.3 B | 38.0±3.5 C | 71.0±5.0 B | 95.0±2.5 A |
| | MGT (h) | 159.8±7.9 A | 130.2±3.4 B | 182.9±6.3 A | 159.2±3.9 A |
| | Puncture force (N) | 0.85±0.03 AB | 0.82±0.06 B | 0.95±0.02 A | 0.91±0.02 AB |
| WT MM | % Germination | 30.0±3.8 a A | 32.0±5.2 a A | 32.0±2.3 a A | 42.0±5.3 a A |
| | MGT (h) | 114.3±7.7 b A | 115.2±7.2 b A | 103.7±9.6 b A | 89.5±7.7 b B |
| | Puncture force (N) | 1.05±0.04 a A | 0.97±0.05 a A | 0.91±0.04 a AB | 0.78±0.04 b B |
| *bs*[4] | % Germination | 19.0±3.8 a AB | 4.0±1.6 b B | 30.0±2.6 a A | 3.0±3.0 b B |
| | MGT (h) | 186.4±2.4 a B | 200.3±7.1 a AB | 178.3±3.1 a B | 228.0±0 a A |
| | Puncture force (N) | 0.87±0.02 b AB | 0.61±0.03 b B | 1.15±0.20 a A | 0.94±0.02 a AB |

**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 *bs*[1]; WT MM versus *bs*[4]). The WT Pieralbo, the *bs*[2] background, is unavailable for comparison.

| Genotype | WT AC | *bs*[1] | *bs*[2] | WT MM | *bs*[4] |
|---|---|---|---|---|---|
| 1000 seed weight (g) | 2.00±0.02 a | 2.57±0.01 b | 3.44±0.02 | 2.09±0.02 a | 2.79±0.02 b |
| Seed area ($mm^2$) | 4.65±0.11 a | 5.76±0.20 b | 6.41±0.14 | 4.78±0.10 a | 5.48±0.11 b |
| Seed number $fruit^{-1}$ | 63.6±10.4 b | 42.8±4.6 b | 101.2±25.8 | 36.8±7.2 a | 40.2±13.0 a |
| Fruit pH | 4.25±0.04 a | 4.19±0.02 a | 4.13±0.04 | 4.07±0.03 b | 4.21±0.06 a |

(Table 2). The pH of the fruit was negatively affected, being greater than that of WT fruit for the *bs*[4] mutant (Table 2). No discernible difference in fruit pH was determined for *bs*[1] relative to WT AC.

Ailsa Craig seeds, both WT and *bs*[1], 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*., 2003*b*), 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*., 2003*b*), 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 *bs*[1] seeds had similar EPR spectra. Neither wild-type Moneymaker nor *bs*[4] exhibited appreciable EPR signals, which is attributed to a lack of unpaired electrons, a hallmark of melanic compounds (Table 3).

### *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 (Gillikin 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*., 2003*b*). The hypothesis was therefore tested that oxygen or a ROS was interacting with some testa component in *bs*[1], *bs*[2], and *bs*[4], 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 *bs*[4] mutants exhibited considerable PRX activity relative to WT (Fig. 3A, B). In-gel activity staining revealed that the *bs*[1] mutant seed also had detectable PRX activity while WT AC did not, but activity assays were unable to document a difference

**Table 3.** *Electron paramagnetic resonance signals from the seeds of* bs*, their WT backgrounds, and two positive control species (niger and sunflower)*

| Sample | No. of scans | Average peak height/ No. of scans |
|---|---|---|
| Niger | 1 | 3520 |
| Sunflower (black) | 1 | 2710 |
| Sunflower (white) | 100 | NAS[a] |
| WT AC | 100 | 414 |
| *bs*$^1$ | 100 | 526 |
| *bs*$^2$ | 100 | NAS[a] |
| WT MM | 100 | NAS[a] |
| *bs*$^4$ | 100 | NAS[a] |

[a] NAS indicates no appreciable signal.

(Fig. 3A, B). Catalase (CAT) activity was statistically significantly greater ($\alpha$=0.05) in *bs*$^1$ *and bs*$^4$ 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 ($\alpha$=0.05) of reactive oxygen species (ROS) were released from intact, dry WT MM seeds than from those of *bs*$^4$ (Fig. 4). This difference was substantially mitigated 24 HAI, but WT MM seeds still released statistically significant greater amounts ($\alpha$=0.05) of ROS into the media than *bs*$^4$ early during the test (Fig. 4). No such statistical difference was apparent, regardless of imbibition status of the seed, for *bs*$^1$ 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*., 1997*a*); and (3) more rapid completion of germination than WT (Atanassova *et al*., 1997*a*, *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 *bs*$^2$×*are*, despite several attempts) and $F_3$ 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).

### *Many ESTs were differentially expressed between WT MM and* bs$^4$ *seeds*

Seeds of the *bs*$^4$ mutants were considerably delayed in radicle protrusion and did not attain the percentage germination of WT MM seeds (Fig. 1; Table 1). Furthermore, *bs*$^4$ 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*., 2003*b*). These aberrations suggested that there might be considerable differences in the transcriptional activity between *bs*$^4$ 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 *bs*$^4$ 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 *bs*$^4$ 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 *bs*$^4$ mutants had greater than usual pH, a phenotype reported for fruit from *bs*$^1$ 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 (*bs*$^4$) 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*. (1997*a*) and Downie *et al*. (2003*b*) could not find a consistent relationship between seed weight and rate of germination for *anthocyaninless* or *blackseed* tomato mutants, respectively. Afterripened *bs*$^1$ mutant seeds attained a final percentage germination comparable with WT AC on both water and 100 µM $GA_{4+7}$ (Fig. 1A, B). These results argue against the poor

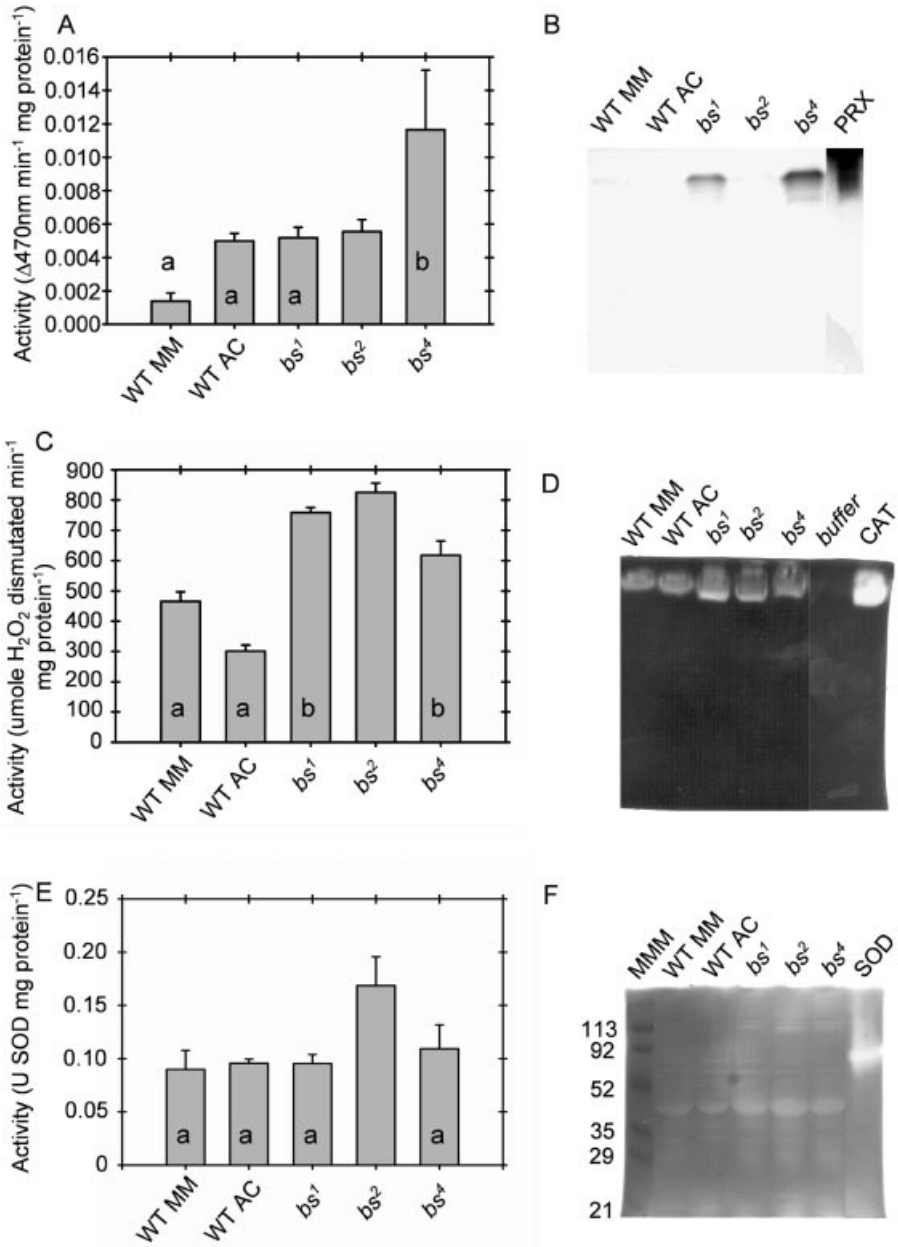



**Fig. 3.** The activity of (A) peroxidase (PRX), (C) catalase, and (E) superoxide dismutase was assayed in mutant and wild-type seeds (the *bs*$^2$ parental line, Pieralbo is unavailable for comparision). Different lowercase letters in or above the bars depicting enzyme activity denote statistically 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.

germination of this mutant being due to the effects of the testa on light quality. The dark pigment in the mutant testa was also unlikely to be a germination inhibitor (Walker, 1962) because, when the testa and endosperm cap opposing the radicle were removed from *bs* 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*$^1$ 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).

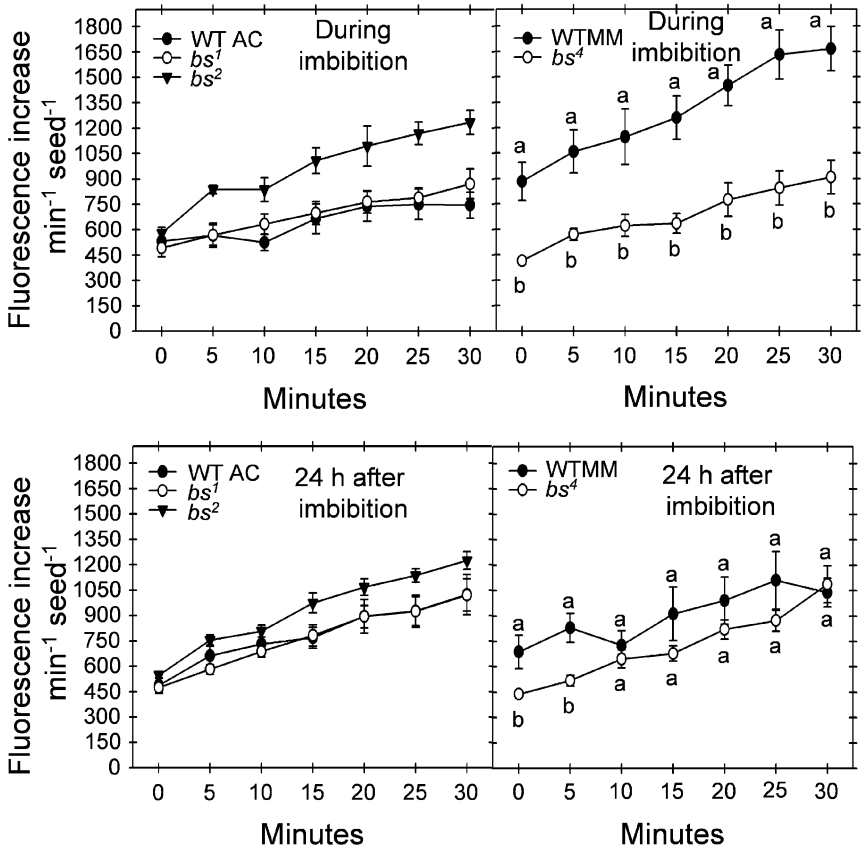



**Fig. 4.** The amount of reactive oxygen species released from mutant and wild-type seeds during or 24 h after imbibition was analysed. Different lower case letters for each time point between the genotypes signify statistically significantly different mean ROS release. There were no significant differences between *bs*[1] and WT AC in ROS release at any time point and so these curves were not lettered.

Many seeds of both WT MM and *bs*[4], 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*., 2003*a*). 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 *bs*[4] seeds on water was also marginally improved by afterripening and a combination of afterripening and imbibition on $GA_{4+7}$ greatly improved *bs*[4] 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*., 2003*b*). One report has suggested that inhibition of CAT is required for dormancy alleviation by redirecting $H_2O_2$ 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 *bs*[1] and/or *bs*[4] 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.

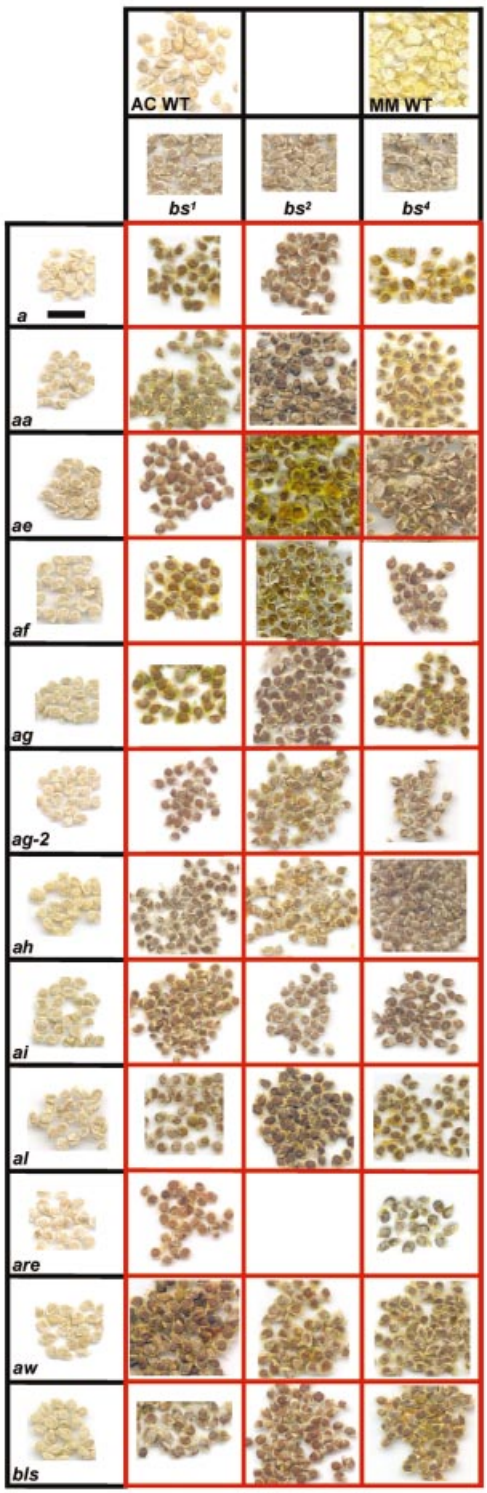



**Fig. 5.** Double mutant seeds between the dark testa mutants and 12 *anthocyaninless* lines were generated. *a*: *anthocyaninless*; *aa*: *anthocyanin absent*; *ae*: *entirely anthocyaninless*; *af*: *anthocyanin-free*; *ag*: *anthocyanin gainer*; *ag-2*: *anthocyanin gainer 2*; *ah*: *Hoffmann's anthocyaninless*; *ai*: *incomplete anthocyanin*; *al*: *anthocyanin loser*; *are*: *anthocyanin reduced*; *aw*: *without anthocyanin*; *bls*: *baby lea syndrome*. The bar in the *anthocyaninless* square depicts 1 cm.

### *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*., 1997*a*) 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*., 2003*b*) 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*., 1997*a*). However, the $bs^{1,2,4}$ 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*., 2003*b*).

### *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*., 2003*b*). The $bs^4$, but not the other mutant seeds, accumulate pigment in the periclinal, secondarily thickened cell walls of the first endosperm cell file (Downie *et al*., 2003*b*), and increased germination percentage in response to 100 μM $GA_{4+7}$. The force required to penetrate the micropylar endosperm and testa was significantly greater for air-dried $bs^1$ and *bks* seeds 24 HAI on water relative to their respective WTs

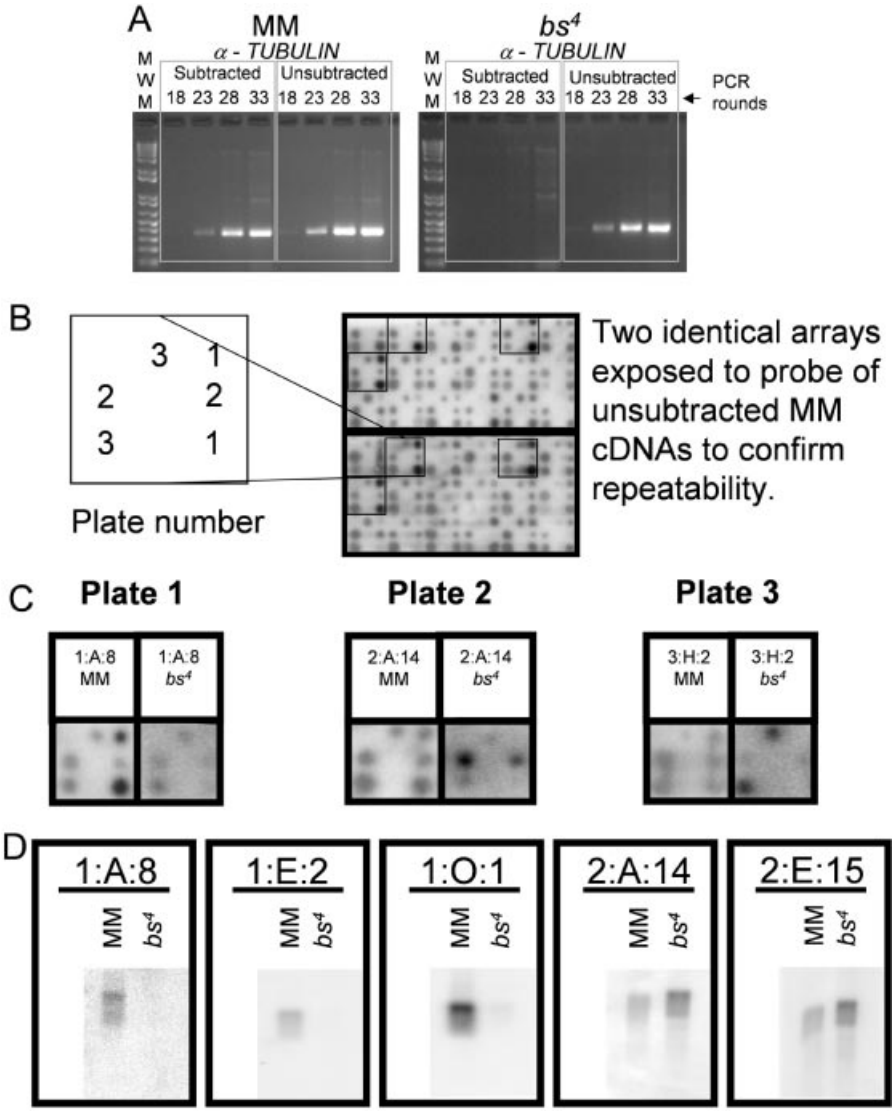



**Fig. 6.** Differential expression between WT MM and $bs^4$. (A) Subtractive, suppressive PCR produced two libraries enriched for transcripts preferentially expressed in WT MM or $bs^4$ seeds. The degree to which the libraries were enriched for differentially expressed transcripts was tested using tubulin-specific 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.

(this report and Downie *et al*., 2003*b*). 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*., 2003*b*). 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 WTs (Fig. 3) and *bks* mutant seeds accumulate free radicals while WT MT did not. In conclusion, the $bs^1$, $bs^4$, 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, suppressive PCR libraries and their differential expression between the two genotypes, confirmed hypotheses that the

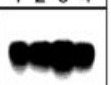
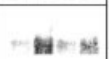
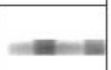
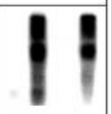
| Accession | Best homologue in the databases | E-value | Library | Northern lanes 1 2 3 4 |
|---|---|---|---|---|
| CF243397 | Y08427 *N.tomentosiformis* internal transcribed spacer between 26S and 18S rRNA genes. | $2e^{-42}$ | $bs^4$ + |  |
| CF243398 | AB048731 Lycopersicon esculentum lec2sa1 gene for 2S seed albumin-1 large subunit | $6e^{-10}$ | $bs^4$ + |  |
| CF243399 | No match | - | WT MM + |  |
| CF243400 | No match | - | $bs^4$ + |  |
| CF243401 | AY079391 *Arabidopsis thaliana* unknown protein (At2g47770) mRNA | $3e^{-37}$ | $bs^4$ + |  |
| CF243402 | AJ298245 *Quercus robur* mRNA for geranyl diphosphate synthase (gppS gene). | $1e^{-38}$ | WT MM + |  |
| CF243403 | *Arabidopsis thaliana* 26S proteasome regulatory subunit (RPN11); NM_122261 similar to 26S proteasome regulatory particle non-ATPase subunit 11 GI:17297983 from Oryza sativa | $9e^{-89}$ | WT MM + |  |
| CF243404 | AY150039 *Lycopersicon esculentum* 17.6 kD class I small heat shock protein (HSP17.6) mRNA, complete cds | $6e^{-86}$ | WT MM + |  |
| CF243405 | Y15813 *Solanum commersonii* mRNA for DHN1 protein, a dehydrin-like gene. | $3e^{-26}$ | $bs^4$ + |  |
| CF243406 | AK103333 *Oryza sativa* (japonica cultivar-group) cDNA clone:J033125O08 | $2e^{-26}$ | $bs^4$ + |  |
| CF243407 | No match | - | $bs^4$ + |  |
| CF243408 | *Zea mays* PCO080072 mRNA sequence. AK117715 *Arabidopsis thaliana* At1g61670 mRNA for unknown protein | $3e^{-09}$ | WT MM + |  |
| CF243409 | U97700 *Sesamum indicum* 15.5 kDa oleosin mRNA | $5e^{-31}$ | $bs^4$ + |  |
| CF358967 | No match | - | WT MM + |  |
| CF358968 | *S.tuberosum* mRNA encoding homolog to human P23 tumor protein | $5e^{-16}$ | $bs^4$ + |  |
| CF358969 | No match | - | $bs^4$ + |  |

**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-imbibed 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.

$bs^4$ 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 $bs^4$ 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*., 2003*b*). These suppositions are all harmonious with the recessive, Mendelian inheritance of the $bs^4$

lesion. Whether the other *bs* mutants or the *bks* mutant also fail to exit the developmental programme is under investigation.

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