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Norfolk Plant Sciences

Information Supporting a Regulatory Status Review of Tomato Genetically Engineered to Produce Increased Levels of Anthocyanins

Norfolk Plant Sciences is submitting this information to support a Regulatory Status Review by the USDA Animal and Plant Health Inspection Service under 7 CFR Part 340.4

Submitted on behalf of:

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Summary

Norfolk Plant Sciences is submitting this information to USDA APHIS to support a Regulatory Status Review of tomato (*Solanum lycopersicum*) that has been genetically engineered to produce enhanced levels of endogenous anthocyanins in the fruit. Norfolk Plant Sciences has introduced into tomato the *Delila* (*Del*) and *Rosea1* (*Ros1*) genes derived from the common garden snapdragon (*Antirrhinum majus*). Both genes are expressed under the tomato fruit specific promoter E8. The *Del* and *Ros1* genes encode transcription activators of anthocyanin biosynthetic genes resulting in increased production of the two major anthocynanins in tomato fruit. These anthocyanins, delphinidin 3-*O*-(coumaroyl) rutinoside-5-*O*-glucoside, and petunidin 3-*O*- (coumaroyl) rutinoside-5-*O*-glucoside, are produced in tomato leaves to protect photosynthetic tissues from light stress and are also found in the skin of tomato fruit resulting from crosses of *S. esculentum* (cultivated tomato) with wild species *S. chilense* or *S. cheesmaniae.* Tomato fruits produced from these crosses are currently available to consumers as the commercial tomato varieties *Indigo Rose* and *Sunblack* (Mes, 2005; Mes *et al*., 2008; Gruber, 2017).

Anthocyanins are red, purple and blue pigments that color many fruits, flowers and some vegetables. Dietary anthocyanins are associated with a reduced risk of chronic and degenerative diseases such as cardiovascular disease, obesity and certain cancers. Dietary anthocyanins have been reported to have protective effects against myocardial infarction and coronary heart disease (Cassidy *et al*., 2013) as well as ameliorative effects in those already at risk of atherosclerosis (Zhu *et al*., 2012). People consuming a high fruit and vegetable diet may eat as much as 60 mg of anthocyanins per day, although beneficial effects required 5-fold higher anthocyanin consumption levels (>300 mg/day) in human intervention studies (Pojer *et al*., 2013). Consequently, anthocyanin levels in the most commonly eaten fruits and vegetables are probably inadequate to confer optimal health benefits. In addition, overall consumption of fruit and vegetables has declined over the last 10 years with only a fraction (~25%) of the U.S. population consuming the recommended 5 daily portions (Potter *et al*., 2000). Anthocyanins occur in almost all higher plants, mostly in flowers and fruits but they are also present in leaves, stems, and some roots and in several familiar fruits and vegetables (Table 1). Anthocyanins are also used as food additives in the EU (EU food additive number E163) and in the U.S. the FDA lists products rich in anthocyanins as approved for use as food colorants.

The major anthocyanins of tomato, delphinidin 3-*O*-(coumaroyl) rutinoside-5-*O*-glucoside and petunidin 3-*O*-(coumaroyl) rutinoside-5-*O*-glucoside (Figure 1) are

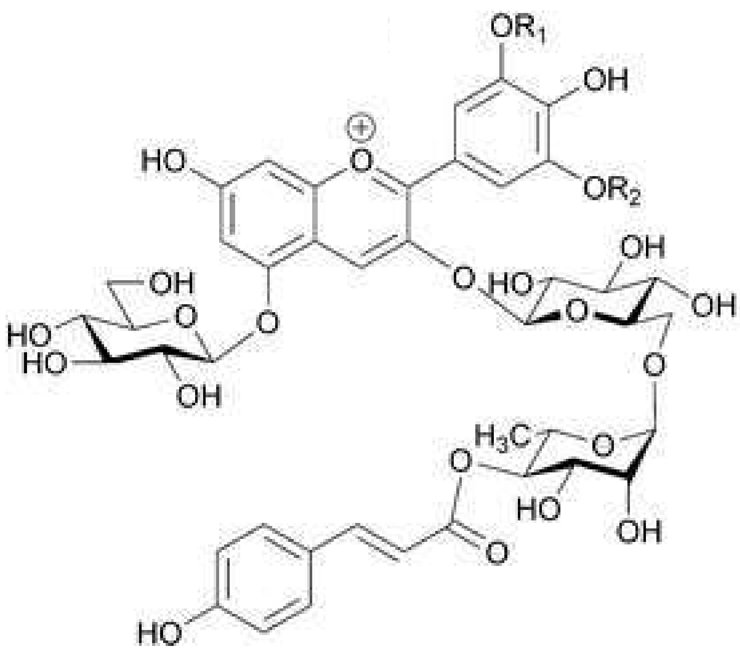
identical to those present in eggplant (Azuna *et al*., 2008) and purple flesh potatoes (Lachman *et al*., 2009). Other common dietary sources of anthocyanins include blood oranges, elderberry, olives, red onion, fig, purple sweet potato and purple corn (Table 1). No reports of adverse effects associated with the consumption of anthocyanins in food have been identified (Pojer *et al*., 2013). Intervention studies supplementing daily anthocyanin consumption with 640 mg anthocyanins per day (Hassellund *et al*., 2012, and 2013) reported no adverse effects, but rather positive effects in elevating HDL-cholesterol levels in healthy males. LD_{50} values for anthocyanins in excess of 2g/kg have been calculated, with no signs of ill effects at these levels (Pojer *et al*., 2013).

The high-anthocyanin tomato variety developed by Norfolk Plant Sciences (referred to herein as the Purple Tomato due to the deep purple color of its fruit) that is the subject of this document is intended for the production of tomatoes for processing and fresh market uses, as well as for the production of tomato juice with enhanced levels of anthocyanins. The elevated levels of anthocyanins in the fruit and juice produced from this tomato result in a deep purple color and represents a new source of dietary anthocyanins for consumers. For the purpose of juice production, the purple tomatoes will be processed using standard industry practices (UNIDO Technology Manual for Small-Scale Processing of Fruit and Vegetables, Vienna 2004) that includes a cold break procedure to preserve the anthocyanin content. Norfolk Plant Sciences has completed a food and feed safety and nutritional assessment of the Purple Tomato that was submitted to the US FDA as part of a Biotechnology Notification Consultation (BNF 178).

Table 1: Anthocyanin content of different foods (Manach, 2004).

Foodstuff	Anthocyanin (mg /100 g FW)	Foodstuff	Anthocyanin (mg /100 g FW)
Eggplant	750	Radish	11-60
Blackberry	83-326	Raspberry	10-60
Blackcurrant	130-400	Red cabbage	125-210
Blueberry	25-497	Red currant	80-420
Cherry	100-400	Red grape	15-375
Chokeberry	200-1000	Red onions	7-21
Cranberry	60-200	Red wine	24- 150
Elderberry	450-1375	Rhubarb	200
Orange	8	Strawberry	15-35
Plum	1-12	**Purple Tomato**	**500**

Figure 1. Structure of the major anthocyanins produced in tomato leaves of WT control plants and in the fruit of Purple Tomatoes.
Delphinidin 3-*O*-(coumaroyl)rutinoside-5-*O*-glucoside also known as Nasunin: R1 and R2 = H;
Petunidin 3-*O*-(coumaroyl)rutinoside-5-*O*-glucoside: R1 = CH3 and R2 = H.



1. Description of *Solanum lycopersicum* (Tomato), the Comparator Plant

Tomato is native to Central America and western South America. The people native to these regions cultivated tomato and seeds were transported to Europe by Spanish explorers in the 1600s. Later tomatoes were introduced in North America by European colonists (Rick, 1978). All of the red-fruited species of tomato (*S. lycopersium, S. pimpinellifolium,* and *S. cheesmani*) are self-pollinating but are sexually compatible with each other. Hybrid varieties of tomato have been developed and are generated by manual crossing techniques. Tomato is not considered to be a weedy species (USDA, 1992) and it is not sexually compatible with plants of the closely related nightshade family.

Tomato is a diploid species with 12 chromosome pairs and the genetics of tomato is well characterized (Tanksley, 1993). Under field conditions in the U.S.A., self-pollination occurs at the high rate of 99% (Currence and Jenkins, 1942). Modern tomato varieties were selected for high fertility that inadvertently resulted in stigmas that are recessed in the anther cone and are therefore not able to receive pollen from outside the flower. While cross-pollination in the field is low (about 1%), when it occurs it is facilitated by pollinating insects such as bees.

On a global scale, tomato represents the largest market for fresh fruit and supports a large tomato processing industry. In 2020 approximately 280,000 acres of tomato were planted in the U.S.A. resulting in the production of 11,312 metric tons of tomatoes (USDA/NASS, 2021). Initially, the tomato variety that was genetically engineered by Norfolk Plant Sciences to enhance anthocyanin production in the fruit was the MicroTom variety. Subsequently the purple fruit trait was transferred to other varieties by Mendelian crossing.

2. Genotype of the Purple Tomato

A. Origin and Mode of Action of the *Del* and *Ros1* Genes

The Purple Tomato was developed by the transformation of the MicrTom tomato variety (*Solanum lycopersicum*) with two genes, *Delila* (*Del*) and *Rosea1* (*Ros1*), derived from the garden snapdragon (*Antirrhinum majus*). The *Del* and *Ros1* genes are fused with the fruit specific promoter E8 from tomato (Deikman *et al*., 1992) in order to achieve expression specifically in the fruit during ripening. The *Del* gene encodes a basic helix-loop-helix transcription factor and the *Ros1* gene encodes an R2R3MYB-related transcription factor (Schwinn *et al*., 2006; Goodrich *et al*., 1992; Butelli *et al*., 2008) and together the products of these genes interact to induce anthocyanin biosynthesis in snapdragon flowers. Transcriptomic and metabolomic analyses of Purple Tomato compared to wild type red tomatoes have revealed that changes in gene expression induced by *Del* and *Ros1* are restricted to genes of general phenylpropanoid metabolism and anthocyanin biosynthesis, as confirmed by comparison of metabolite contents (Butelli *et al*., 2008; Zhang *et al*., 2013; Tohge *et al*., 2015).

Transgenic tomato plants containing the *Del* and *Ros1* genes were produced at the John Innes Centre (Norwich, UK). The transformation procedures and results of this work have been reported in detail (Butelli *et al*., 2008). Briefly, tomato leaf discs of the MicroTom variety were transformed using *Agrobacterium tumefaciens* strain LBA4404 containing the binary vector pDEL.ROS containing the *Del* and *Ros1* genes whose expression is under the control of the fruit-specific E8 promoter from tomato. In addition to the *Del* and *Ros1* genes, the *nptII* gene derived from the bacterial transposon Tn5 was also included within the T-DNA of plasmid pDEL.ROS to provide a means of selection of the tomato transformants. The genetic elements contained in the T-DNA of the transformation construct pDEL.ROS (NCBI Accession Number MN580094) are listed in Table 2 and a diagram of plasmid pDEL.ROS is depicted in Figure 2.

B. Origin and Mode of Action of the *nptII* Gene

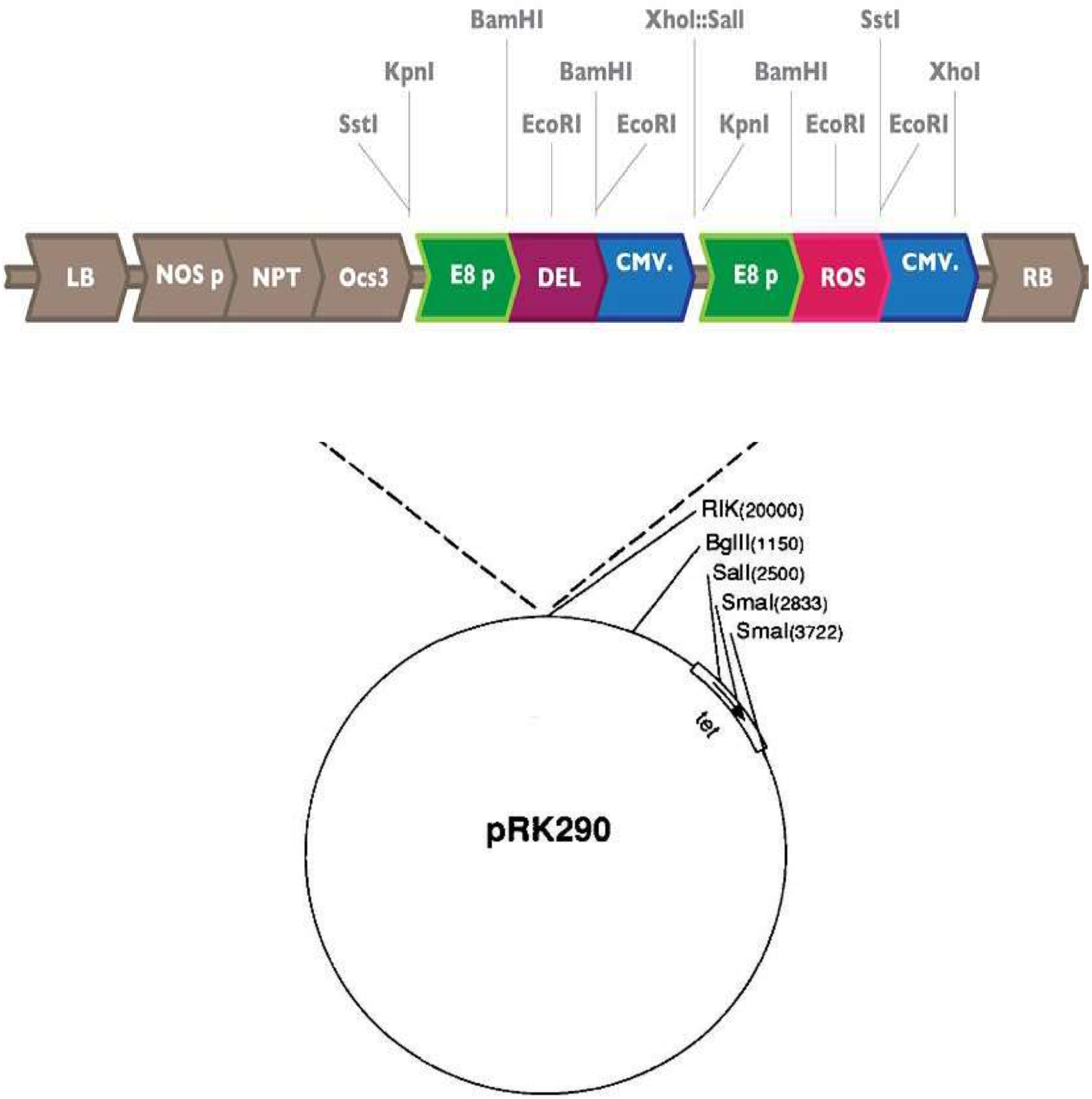
The *nptII* gene derived from the bacterial transposon Tn5 and encoding neomycin phosphotransferase was included in the T-DNA of plasmid pDEL.ROS as a selectable marker gene. Neomycin is lethal to many bacteria and plant cells in culture due to its binding to ribosomes and interference with the translation process such that nascent proteins are misfolded and nonfunctional (Davis, 1988). The neomycin phosphotransferase encoded by the *nptII* gene catalyzes the phosphorylation of neomycin thereby inactivating it and preventing its binding to ribosomes (van den

Elzen *et al*., 1985). Expression of the *nptII* gene in pDEL.ROS is controlled by the promoter from the nopaline synthase (*nos*) gene and the transcription terminator of the octopine synthase gene that were both derived from *Agrobacterium tumefaciens*. The *nos* gene promoter is widely active in the cells of many plants (Depicker *et al*., 1982) and the *nptII* gene has been widely used as a selectable marker for plant transformation. After transformation of tomato with pDEL.ROS, transformed cells containing the *nptII* gene of the T-DNA were selected in culture by the inclusion of neomycin in the culture medium. However, during cultivation in soil or in the environment where neomycin is absent, expression of the *nptII* gene provides no obvious phenotype or selective advantage to the transformed tomato plants. The neomycin phosphotransferase has been shown to be safe for consumption by humans and animals (Nap *et al*., 1992). The *nptII* gene has been used as a selectable marker gene in other varieties of genetically engineered tomatoes that have been deregulated in the U.S. (Deregulation Petitions 94-228-01p, 94-290-01p, 95-053-01p, 95-324-01p, and 97-287-01p) and the *nptII*/tomato trait/crop combination is listed as not regulated by USDA on the USDA Plant-Trait-Mechanism of Action List (USDA, 2021).

Table 2. Description of the genetic elements in the T-DNA of the 33,236 bp transformation construct pDEL.ROS (NCBI Accession Number MN580094.1) that were transformed into the tomato genome.

Genetic Element	Description (Accession Number)	Donor Organism	Function	Reference
LB	Left T-DNA Border (KY000061.1)	*Agrobacterium tumefaciens*	T-DNA boundary	Zambryski *et al.*, 1982
NOSp	Nopaline synthase promoter region (MK439385.1)	*Agrobacterium tumefaciens*	Transcriptional Promoter	Jones *et al.*, 1992
NPT II	Neomycin phosphotransferase gene conferring resis-tance to Kanamycin (U00004.1)	Bacterial transposon Tn5 from *Escherichia coli*	Selectable marker gene; resistance to neomycin antibiotic	van den Elzen *et al.*, 1985
Ocs 3	Octopine synthase termination region (CP033030.1)	*Agrobacterium tumefaciens*	Transcriptional terminator/poly-adenylation signal	Jones *et al.*, 1992
E8p	E8 promoter region (KJ561284.1)	*Solanum lycopersicum*	Transcriptional Promoter	Butelli *et al*, 2008
DEL	*Delila* gene cDNA from snapdragon (M84913.1)	*Antirrhinum majus*	Transcriptional Promoter	(Goodrich *et al.*, 1992)
CMV	Cauliflower mosaic virus termination region (KJ716236.1)	Cauliflower mosaic virus	Transcriptional terminator/ poly-adenylation signal	Hellens *et al.*, 2000
E8p	E8 promoter region from tomato (KJ561284.1)	*Solanum lycopersicum*	Transcriptional Promoter	Butelli *et al*, 2008
ROS	*Rosea1* cDNA from snapdragon (DQ275529.1)	*Antirrhinum majus*	Transcriptional Promoter	Schwinn *et al.*, 2006
CMV	Cauliflower mosaic virus termination region (KJ716236.1)	Cauliflower mosaic virus	Transcriptional terminator/ poly-adenylation signal	Hellens *et al.*, 2000
RB	Right T-DNA Border (KY000061.1)	*Agrobacterium tumefaciens*	T-DNA boundary	Wang *et al.*, 1984

Figure 2. Genetic map of the pDEL.ROS plant transformation vector with the T-DNA component containing the *Del* and *Ros1* genes. pDEL.ROS was constructed by inserting the *Del*/*Ros1* T-DNA cassette shown at the top of the figure into bacterial plasmid pRK290 (shown at the bottom). A description of the individual genetic elements is presented in Table 2.



3. Sequence of the Del/Ros1 T-DNA and the Flanking Tomato Genome

The complete genome sequence of a *Del/Ros1* tomato plant of the MicroTom variety that was six generations advanced from the T_0 transformant was determined by BGI, a publicly listed commercial genomics company in Shenzhen, Guangdong, China. BGI used Illumina HiSeq X Ten sequencing that gave greater than 60-fold coverage of the entire Purple Tomato genome. The genome sequence of the *Del/Ros1* Purple Tomato has been deposited as a BAM file in the European Nucleotide Archive (ENA) under accession number ERR3500875. Examination of the genome sequence revealed the presence of a single insertion of the *Del*/*Ros1* T-DNA located in tomato chromosome 4. The sequence also revealed that a deletion of 94 bp of the tomato genome occurred upon insertion of the T-DNA with additional short deletions on each end of the T-DNA equal to 52 bp at the RB and 75 bp at the LB (Figure 3). Further, exanimation of the genome sequence of the Purple Tomato also demonstrated the absence of any pDEL.ROS sequences derived from outside of the T-DNA element. The annotated nucleotide sequence of the T-DNA region of the Purple Tomato is presented in Figure 4.

Figure 3. Schematic representation of the *Del/Ros1* T-DNA insert in the chromosome of the Purple Tomato.

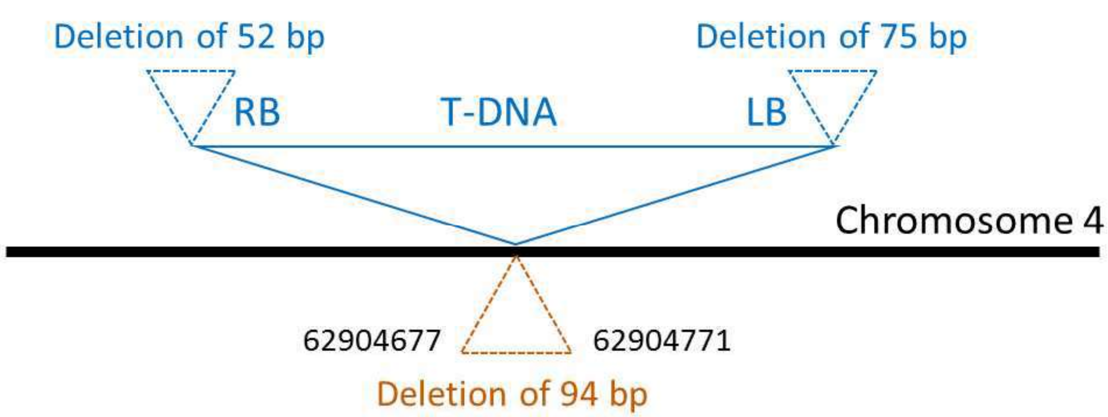


Figure 4. The Annotated Nucleotide Sequence of the Del/Ros1 T-DNA Locus in Tomato.

A. Annotation of the *Del/Ros1* T-DNA insertion. Abbreviations are as listed in Table 2. An overall representation and orientation of the *Del/Ros1* T-DNA insert is represented in Figure 3.

Nucleotide position		Genetic Element
Start	End	
1	332	Tomato chromosomal DNA ("chr4") position 62904346…62904677 (Build SL 3.0). 94 bp of genomic sequence after position 62904677 have been deleted
333	864	RB region of the T-DNA insert 52 bp at the end of the RB region have been deleted (including the RB)
865	1502	**CMV** (Cauliflower mosaic virus termination region)
1615	2277	**ROS** (*Rosea1* cDNA from snapdragon)
2284	4470	**E8p** (E8 promoter region from tomato)
4500	5227	**CMV** (Cauliflower mosaic virus termination region)
5287	7221	**DEL** (*Delila* cDNA from snapdragon)
7305	9493	**E8p** (E8 promoter region from tomato)
10535	11242	**Ocs 3** (Octopine synthase termination region)
11269	12063	**NPT II** (Neomycin phosphotransferase gene conferring resistance to Kanamycin)
12150	12329	**NOSp** (Nopaline synthase promoter region)
12330	12915	LB region of the T-DNA insert 75 bp at the end of the LB region have been deleted (including the LB)
12916	13098	Tomato chromosomal DNA ("chr4") position 62904771…62904953 (Build SL 3.0)

B. Nucleotide sequence of the *Del/Ros1* T-DNA locus including tomato genomic flanking DNA. The annotations of the different genetic elements included in the sequence are listed in the table in Figure 4, A above.

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>Del_Ros1 T-DNA locus including flanking DNA from the tomato genome
atacgtaaaagggtcaaatattattattaaatttttagaaaaatagattaagaaaataaatgaataagtaggtaagtcg
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4. Phenotype of the Purple Tomato

Expression of the *Del* and *Ros1* genes under control of the E8 promoter in tomato fruits results in elevated production of endogenous tomato anthocyanins. As a result of the elevated levels of anthocyanins, the fruit develop a deep purple color (Figure 5). Up until the maturation of the fruit, the Purple Tomato plants grow and develop in a manner identical to conventional tomato varieties. The purple color of the fruit of the *Del/Ros1* tomato extends completely through the flesh of the fruit and is not confined solely to the skin (Figure 6). The anthocyanin content of purple fruits from a hemizygous *Del/Ros1* plant averaged approximately 500 mg per 100 g fresh weight whereas anthocyanins were undetectable in wildtype red tomatoes.

Figure 5. (A) Comparison of the growth and development of a *Del/Ros1* tomato (left) in the MicroTom variety with a nontransgenic wildtype MicroTom tomato (right). (B) Fruit derived from the wildtype MicroTom (top) compared to fruit from the Purple Tomato (bottom) harvested at the mature green (left), breaker (second from left), breaker plus 2 days (third from left), and red ripe (right) stages.

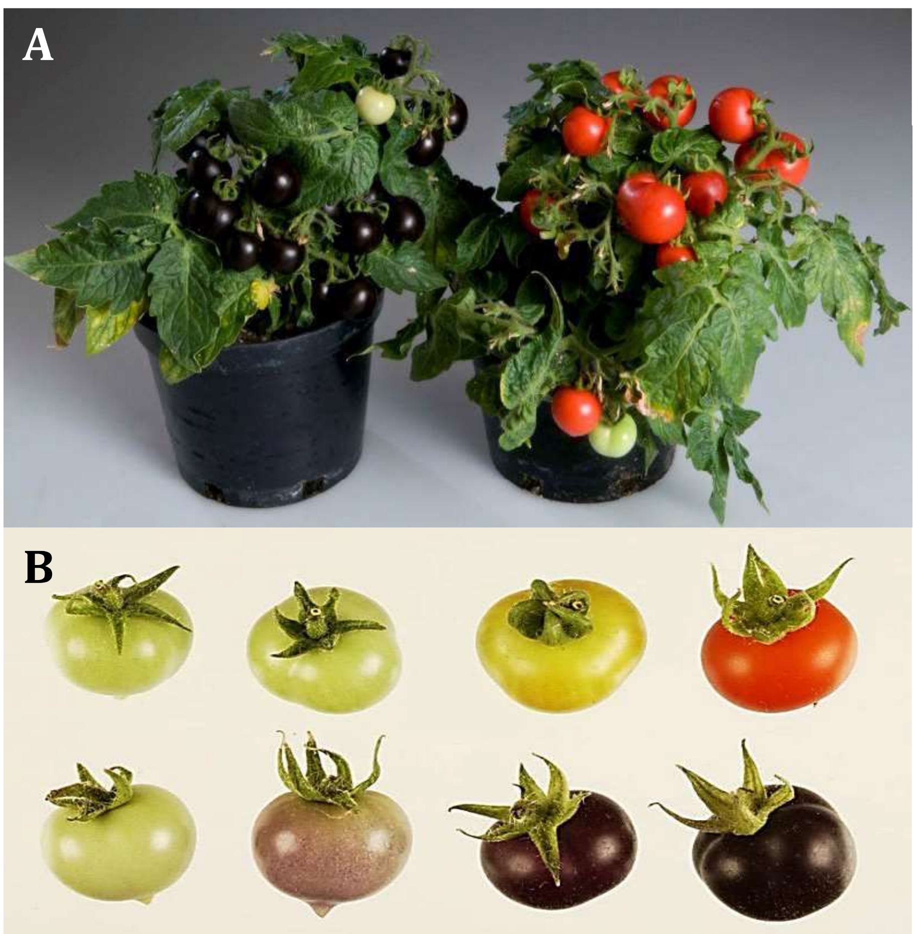

Figure 6. (a) Whole and halved *Del/Ros1* (purple) and wildtype red tomato fruit. (b) Whole and halved ripe red wildtype (top) and *Del/Ros1* (bottom) tomato fruit.

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