'THE LATEST IN SEED COAT FASHION': seed colour (proanthocyanidin) and trichome mutations in a new population of activation-tagged Arabidopsis lines

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Abstract

Flavonoids comprise a diverse group of phenolic compounds, which serve a variety of ecological and physiological functions in plants. These include attraction of pollinators, defense against predators and pathogens, and protection against UV damage. Flavonoid polymers (proanthocyanidins, also known as condensed tannins) are advantageous for controlling ruminant digestion and insect foraging. They also have wide-ranging benefits in human health. In the oilseed and vegetable Brassicas, they are present in brown and black seed coats as a part of the anti-nutritional phenolic fibre fraction and their reduction is the focus of major breeding programmes around the world. Several collections of chemically-induced and *T-DNA*-induced mutants in the Arabidopsis flavonoid pathway have been screened worldwide to find *transparent testa* (*tt*) and *tannin-deficient seed* (*tds*) mutants in the proanthocyanidin pathway. These studies have led to a detailed understanding of the enzymology and metabolic regulation of much of the flavonoid pathway. Seventeen flavonoid genes have been characterized over the past decade in Arabidopsis, including six regulatory genes, a vacuole transporter, and eleven structural genes, but genes defining the last steps in polymer formation are unknown. Recently, a new population of activation-tagged Arabidopsis lines (~50,000) and *T-DNA* -tagged lines (~20,000) were developed in Saskatoon, Canada. Within this population, we have discovered additional seed coat colour alleles, interacting trichome alleles, and several novel proanthocyanidin-free mutants. The lines are being used to re-define the pathway and as a basis for isolating novel genes.

Key words: Arabidopsis, proanthocyanidin, transparent testa, tannin-deficient seed, mutants

Introduction

Flavonoids comprise a diverse group of phenolic compounds, which serve a variety of ecological and physiological functions in plants. These include attraction of pollinators, defense against predators and pathogens, and protection against UV damage. Flavonoid polymers (proanthocyanidins, also known as condensed tannins) are advantageous for controlling ruminant digestion and insect foraging. They also have wide-ranging benefits in human health. In the oilseed and vegetable *Brassicas*, they are the pigments present in brown and black seed coats as a part of the anti-nutritional phenolic fibre fraction. Their reduction is the focus of major yellow seed breeding programmes around the world (reviewed in Marles et al., 2003).

Several collections of chemically-induced and *T-DNA*-induced mutants in the Arabidopsis flavonoid pathway have been screened worldwide to find *transparent testa* (*tt*) and *tannin-deficient seed* (*tds*) mutants in the proanthocyanidin pathway (Fig. 1) (Abrahams et al., 2002; Shikazono et al., 2003; Kitamura et al., 2004; reviewed in Marles et al., 2003). These studies have led to a detailed understanding of the enzymology and metabolic regulation of much of the flavonoid pathway. At least 15 flavonoid genes have been characterized over the past decade in Arabidopsis (Fig. 1), but genes defining the last steps in polymer formation are unknown.

Recently, a new population of activation-tagged Arabidopsis lines (~50,000) and *T-DNA* -tagged lines (~20,000) were developed in Saskatoon, Canada (Weigel et al., 2000). Within this population, we have screened for proanthocyanidin-reduced and proanthocyanidin-overproduced seed coat mutants.

Materials and Methods

Dry mature seeds were screened visually for changes in seed color T3 lines of a new Arabidopsis T-DNA and activation-tagged population (containing 4 tandem 35S enhancers) (Weigel et al., 2000). Lines were classified into yellow, gray, pale brown, red and black candidate seed mutants , then screened histochemically for proanthocyanidin and flavan-3-ols using three methods: dimethylaminocinnamaldehyde (DMACA), butanol-HCL, and vanillin-HCL (reviewed in Marles et al., 2003). Selected lines were self-pollinated and propagated for three consecutive generations to check the stability of the visual phenotype, then stained again histochemically to identify the proanthocyanidin phenotype of the T4, T5 and T6 generations. Growth variation and anthocyanin accumulation in vegetative tissues were observed after nutrient deficiency, flooding, and transplantation, steps which can detect pleiotrophic phenotypes. Lines were also observed for abnormalities in trichome morphology and density, since trichomes can be affected by regulatory mutations in the flavonoid pathway (Walker et al., 1999; Baudry et al., 2004).

Phenotype-stable lines were crossed with each other, sorted into complementation groups, crossed with published

PA-deficient *transparent testa* (*tt*) and *tannin deficient seed* (*tds*) mutants with similar or identical histochemical staining patterns, and back-crossed to two wild type cultivars (Columbia and Landsberg). Lines allelic to published mutants were set aside unless they displayed a novel seed or growth phenotype. Attention was focused at finding new mutations that characterized the lower steps of the flavonoid pathway and novel regulatory mechanisms. Seed colour intensity and DMACA staining patterns were compared between short-term and long-term storage of novel and published lines.

DNA sequence of T-DNA flanking regions was determined in stable T4 phenotypes by modified TAIL-PCR and plasmid rescue (Liu et al., 1995; Sessions et al., 2002). Sequence obtained by these two methods was blasted against several databases. The Arabidopsis genome was surveyed for 10kb on either side of the disrupted gene to search for regulatory genes and homologues to proanthocyanidin biosynthetic genes, which may have been up-regulated by the enhancers. Southern blot analysis was conducted with probes to several different parts of the T-DNA to find the number of insertions within each novel line. Mapping populations were developed when the T-DNA mutation could not be successfully assigned to a specific gene sequence or when spontaneous mutations were recovered. Complementation of the mutant with a wild-type Arabidopsis gene and recreation of the mutant phenotype using RNAi were used to confirm the molecular basis of the mutant phenotype. Northern blotting was conducted on RNA extracted from different organs of ecotype Columbia and proanthocyanidin mutant lines, including roots, leaves, stems, buds, flowers, and developing siliques. Plant tissues were also ground in liquid N_2 and extracted for anthocyanin, flavonoids and proanthocyanidin by standard methods (Harborne, 1998; Markham, 1982; Porter et al., 1986; Abrahams et al., 2002).

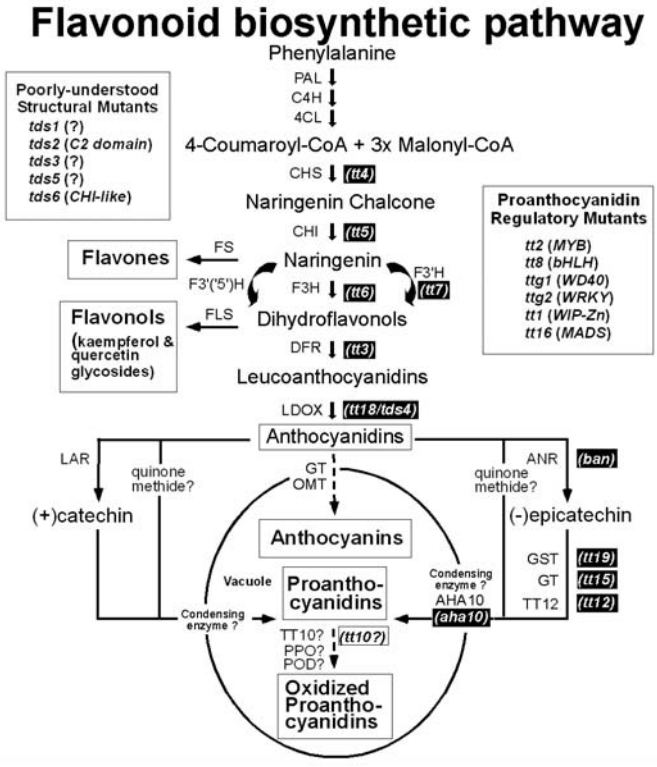

Figure 1. Steps in the proanthocyanidin pathway and proteins affected in published Arabidopsis *tt* and *tds* mutants.

Structural gene-encoded proteins:

CHS (*tt4*): Chalcone Synthase

CHI (*tt5*): Chalcone Isomerase

F3H (*tt6*): Flavonol 3-hydroxylase

F3′H (*tt7*): Flavonoid 3′-hydroxylase

F3′(5′)H: Flavonoid 3′(5′) hydroxylase (not found in Arabidopsis)

DFR (*tt3*): Dihydroflavonol 4-reductase

FS: Flavone synthase (not found in Arabidopsis)

FLS: Flavonol synthase

LDOX=ANS (*tt18=tds4*): Anthocyanin synthase (leucoanthocyanidin dioxygenase)

BANYULS (ANR) (*ban*): Anthocyanin reductase

GST-like (*tt19*): Glutathione *S*-transferase

GT (*tt15*): Glycosyl transferase

TT12 (*tt12*): PA precursor vacuolar membrane transporter (MATE)

AHA10 (*aha10*): auto-inhibited H^+ ATPase LAR: leucoanthocyanidin reductase (not found in Arabidopsis)

TT10 (tt10): laccase oxidation of flavonols and flavan-3-ols

POD: peroxidase (involvement not clear)

PPO: polyphenol oxidase (involvement not clear)

Condensation step may need a condensing enzyme.

Regulatory transcriptional factors (TF)

TT2 (*tt2*): MYB TF

TT8 (*tt8*): bHLH TF

TTG1 (*ttg1*): WD40 TF

TTG2 (ttg2): WRKY TF

TT1 (*tt1*) WIP zinc finger TFs

TT16 (*tt16*): MADS domain TF

Results and Discussion

More than 20 lines were recovered from screening 70,000 T3 lines of the Arabidopsis population for altered seed colour phenotypes. In addition to the brown seed phenotype of the Columbia parental line, seed colour patterns within the population included solid colour phenotypes (pale-yellow, lemon-yellow, pale red-brown, deep red-brown, dark-tan, pale-brown, brown, deep-brown), spotted phenotypes (small dark spots on a solid yellow background), and dark patchy phenotypes (Fig. 2). DMACA distinguished phenotypes in more detail than vanillin or butanol:HCl histochemical staining. In spotted and patchy lines, the dark areas of the seed surface were more focused and smaller without staining than the identical areas stained with DMACA.

Most lines were allelic to published *tt* and *tds* mutants, although some of these alleles showed more intense anthocyanin coloration under stress conditions than the published mutants. Five novel phenotypes were recovered. Three novel lines showed unique dark patchy patterns. A grey-like colour was found on the seed coats of one of the novel patchy mutants, as well as on *tt9* and *tt15* lines. One novel seed line had an unstained mature seed colour very close to the brown color of ecotype Columbia seeds. DMACA staining of this line indicated no proanthocyanidins or flavan-3-ols, suggesting that a non-PA brown seed pigment has accumulated in the seed coat of this mutant.

Figure. 2. Dark and red proanthocyanidin lines within the SRC Arabidopsis mutant population. Ecotypes Columbia (WTC), Landsberg erecta (Ler) and WS have brown coloured seed coats.

One of the novel phenotypes was allelic to *ttg2* in diallelic crosses, but displayed a novel trichome phenotype. Trichomes were tri-branched, but the branches were raised upward parallel to the stalk, instead of at the horizontally angled positions characteristic of Columbia trichomes (Fig. 3). Trichome density in this novel phenotype was very close to the Columbia phenotype, whereas the *ttg2* allele had very few trichomes.

Figure 3. Trichome phenotypes of ecotype Columbia (left), *ttg2* (middle) and an allelic mutant recovered from the SRC Arabidopsis mutant population (right).

A darkening phenomenon was observed during longer term storage of ecotype Columbia seed and several *tt* and *tds* lines. These lines included *tt6*, *tt7*, *tt10*, *tt15*, *tt18*, *tt19*, *tds,* and one of the novel mutant lines (Fig. 3). Non-darkening Arabidopsis lines included *tt3*, *tt4*, *tt5*, *tt12*, *aha10*, and all known seed coat pigment regulatory mutants.

Fig. 3. Illustration of a proanthocyanidin-free Arabidopsis line (*tt18*) (left) which has darkened during long term storage (right).

Most of the SRC lines of Arabidopsis had more than one T-DNA insert. Insertion patterns included tandem T-DNA repeats and single insertions coupled with inverted T-DNA repeats or direct repeats in which a plasmid backbone was included. Several of the mutants were disrupted in novel unknown genes. A few lines are being mapped to determine the molecular basis of the mutation. One is disrupted in a glutathione S-transferase gene, but the phenotype is unique compared with published GST mutations in Arabidopsis.

Conclusions

We have screened a new population of 70,000 Arabidopsis mutations and discovered novel proanthocyanidin seed coat phenotypes, pleitrophic trichome alleles, and several novel proanthocyanidin-free mutants. The lines are being used as a basis for isolating novel genes that define the proanthocyanidin pathway.

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