Oxydation des flavonoïdes dans les graines d’Arabidopsis thaliana. Analyse fonctionnelle du gène TRANSPARENT TESTA 10 codant une polyphénoloxydase de type laccase

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Oxydation des flavonoïdes dans les graines d'*Arabidopsis thaliana*. Analyse fonctionnelle du gène *TRANSPARENT TESTA 10* codant une polyphénoloxydase de type laccase
Life, sometimes so wearying
Is worth its weight in gold
The experience of traveling
Lends a wisdom that is old
Beyond our 'living memory'
A softly spoken prayer:

"It's the journey that's important,
Not the getting there!"

[...]

By John McLeod
Acknowledgments

After more than three years of the journey through the PhD, the time has come to look back and acknowledge all the people I have shared this part of the life with.

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I dedicate this thesis to my parents Jadwiga and Leon.
Abstract

Title:

Flavonoid oxidation in Arabidopsis thaliana seeds. Functional analysis of the TRANSPARENT TESTA 10 gene encoding a polyphenoloxidase of the laccase type.

Abstract: Arabidopsis seeds accumulate flavonoids (proanthocyanidins and flavonols) during their development. A previous study has shown that a laccase (AtLAC15) encoded by the TRANSPARENT TESTA 10 (TT10) gene could trigger flavonoid oxidation in the seed coat. If both proanthocyanidins (PAs) and flavonols appear to be TT10 protein substrates, only PA oxidation leads to brown pigments responsible for the mature seed coat color. An important consequence of TT10 activity on seed flavonoid metabolism is an increased ratio of insoluble to soluble PAs. The physiological functions of TT10 are still unknown, however defense against biotic and abiotic stresses, either constitutive or induced, may be predicted on the basis of present knowledge on polyphenoloxidases.

The purpose of this thesis was to perform a functional characterization of the TT10 gene. A part of the work was devoted to the analysis of the regulatory mechanisms controlling the developmental pattern of TT10 gene expression in seeds and vegetative plant parts. The functional 5’-dissection of a 2.0-kb promoter realized with the uidA reporter gene encoding β-glucuronidase (GUS) was performed to identify regions responsible for activation in seed and other plant organs. TT10 promoter happens to be activated exclusively in seed coat and siliques. Directed mutagenesis was undertaken to precise the regulatory role of in silico-detected cis-acting regulatory elements (CAREs) located in a 194-bp region necessary for expression in seed coat. TT10 gene expression assessed in different tissues at various stages of development using qRT-PCR matched promoter activity pattern. Natural variation for TT10 expression among Arabidopsis accessions was also detected, with the levels of TT10 mRNA in Cvi, Ler and Sha being strongly reduced compared to the ones in Ws, Col and Bay. The impact of this molecular polymorphism on seed flavonoid composition, as analyzed on mature seeds with LC-MS, is discussed. In silico analysis of the TT10 promoter revealed the presence of putative CAREs potentially involved in signaling and response to biotic and abiotic stresses. However histochemical analysis of GUS activity in transgenic Arabidopsis plantlets expressing pTT102.0−kb : GUS failed to detect any ectopic activity when submitted to a variety of stresses. This result suggests that transcriptional response to environmental stimuli is highly constrained by developmental parameters. TT10 appeared to be the only member of the laccase gene family to be strongly expressed in seeds. TT10 function may have evolved towards flavonoid oxidation by co-localization with these substrates, which is ensured by tissue-specific gene expression.

Key words: Arabidopsis, flavonoids, gene, laccase, mutant, promoter, seed, transcriptional regulation

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Résumé

Titre:

Oxydation des flavonoïdes dans les graines d’Arabidopsis thaliana. Analyse fonctionnelle du gène TRANSPARENT TESTA 10 codant une polyphénoloxydase de type laccase.

Résumé:

Les graines d’Arabidopsis accumulent des flavonoïdes (proanthocyanidines et flavonols) durant leur développement. Une étude précédente a montré qu’une laccase (AtLAC15) codée par le gène TRANSPARENT TESTA 10 (TT10) induisait l’oxydation des flavonoïdes au niveau des téguments. Si les proanthocyanidines (PAs) et les flavonols sont des substrats de la protéine TT10, seule l’oxydation des PAs conduit à la formation de pigments bruns responsables de la couleur de la graine mature. Une importante conséquence de l’activité de TT10 sur les flavonoïdes de la graine est l’augmentation du rapport PAs insolubles / PAs solubles. Les fonctions physiologiques de TT10 sont toujours inconnues, mais un rôle dans la défense contre des stress biotiques et abiotiques constitutifs ou induits peut être prédit sur la base des connaissances actuelles sur les polyphénoloxydases.

L’objectif de cette thèse était de réaliser une analyse fonctionnelle du gène TT10. Une partie de l’étude était consacrée à l’analyse des mécanismes de régulation contrôlant le pattern développemental d’expression du gène TT10 dans les graines et les parties végétatives. Une dissection fonctionnelle en 5’ d’un promoteur de 2.0-kb réalisée à l’aide du gène rapporteur uidA codant la β-glucuronidase (GUS) a été réalisée pour identifier les régions responsables de l’activation du promoteur dans les graines et les autres parties de la plante. Le promoteur de TT10 est activé exclusivement dans les téguments de la graine et les siliques. Une mutagénèse dirigée a été réalisée dans une région de 194 pb nécessaire à l’expression dans les téguments, pour préciser la fonctionnalité des éléments régulateurs agissant en cis (ERACs) identifiés in silico. L’expression du gène TT10 mesurée par qRT-PCR dans différents tissus et à différents stades de développement est en accord avec le pattern d’activité du promoteur. Une variation naturelle pour l’expression de TT10 a aussi été détectée parmi plusieurs accessions d’Arabidopsis, avec des niveaux d’ARNm mesurés chez Cvi, Ler et Sha fortement réduits par rapport à ceux relevés chez Ws, Col et Bay. L’impact de ce polymorphisme moléculaire sur la composition en flavonoïdes de la graine, analysée sur graines matures par LC-MS, est discuté. L’analyse in silico du promoteur de TT10 a révélé la présence d’ERACs potentiellement impliqués dans la signalisation et la réponse aux stress biotiques et abiotiques. Cependant l’analyse histochimique de l’activité GUS de plantes transgéniques exprimant pTT102.0−kb : GUS n’a pas permis de détecter d’activité ectopique en présence de stress variés. Ce résultat suggère que la réponse transcriptionnelle aux stimuli environnementaux est fortement conditionnée par les paramètres développementaux. TT10 est le seul membre de la famille des laccases à être exprimé fortement dans les graines. La fonction de TT10 a probablement évolué vers l’oxydation des flavonoïdes par la co-localisation avec ces substrats, qui est assurée par la spécificité tissulaire d’expression du gène.

Mots clés: Arabidopsis, flavonoïdes, gène, graine, laccase, mutant, promoteur, régulation transcriptionnelle

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Abbreviations

# number, clone
A adenosine
ATP adenosine-5'-triphosphate
aa amino acid
ABA abscisic acid
AGI arabidopsis genome initiative
attB1, attB2 recombination sites in GATEWAY® cloning
BAC Bacterial Artificial Chromosome
BAR Bio-Array Resource
Bay Bayreuth
bp base pairs
BSA Bovine Serum Albumin
C cytidine
CARE(s) Cis-Acting Regulatory Element(s)
cDNA complementary DNA
CDS coding sequence
Col Columbia
Cvi Cape Verde Islands
DAF Days After Flowering
DMF N,N-dimethylformamide
DMSO dimethylsulfoxide
DNA deoxyribonucleic acid
DNase deoxyribonuclease
dNTP deoxyribonucleotide triphosphate
°C degrees Celsius
DTT dithiothreitol
EC epicatechin
EDTA ethylene diamine tetraacetic acid
EF1 ELONGATION FACTOR 1aA4
eFP electronic fluorescent pictograph
e.g. exempli gratia (for example)
EMS ethane methyl sulfonate
EST expressed sequence tag
EtOH ethanol
g gram
G guanosine
GA gibberellic acid
gcRMA GeneChip Robust Multiarray Averaging
gDNA genomic DNA
GFP green fluorescent protein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>GL</td>
<td>GLABRA</td>
</tr>
<tr>
<td>GUS</td>
<td>β-glucuronidase</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>HIS</td>
<td>histidine</td>
</tr>
<tr>
<td>ii</td>
<td>inner integument</td>
</tr>
<tr>
<td>INRA</td>
<td>French National Institute for Agricultural Research (fr. l’Institut National de la Recherche Agronomique)</td>
</tr>
<tr>
<td>kb</td>
<td>kilo base</td>
</tr>
<tr>
<td>l</td>
<td>liter</td>
</tr>
<tr>
<td>LAC</td>
<td>laccase</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani medium</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography coupled with mass spectrometry detector</td>
</tr>
<tr>
<td>Ler</td>
<td>Landsberg erecta</td>
</tr>
<tr>
<td>μ</td>
<td>micro</td>
</tr>
<tr>
<td>m</td>
<td>meter</td>
</tr>
<tr>
<td>M</td>
<td>mega</td>
</tr>
<tr>
<td>M</td>
<td>molar concentration (mole/liter)</td>
</tr>
<tr>
<td>MCS</td>
<td>multi cloning site</td>
</tr>
<tr>
<td>mm</td>
<td>millimeter</td>
</tr>
<tr>
<td>MPI(K)</td>
<td>Max Planck Institute (Cologne)</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>n</td>
<td>nano</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>oi</td>
<td>outer integument</td>
</tr>
<tr>
<td>o/n</td>
<td>over night</td>
</tr>
<tr>
<td>PA(s)</td>
<td>proanthocyanidins (condensed tannins)</td>
</tr>
<tr>
<td>p</td>
<td>promoter</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pH</td>
<td>negative logarithm of the proton concentration</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real time - PCR</td>
</tr>
<tr>
<td>RIL(s)</td>
<td>recombinant inbred line(s)</td>
</tr>
<tr>
<td>RMA</td>
<td>Robust Multiarray Averaging</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase H</td>
<td>ribonuclease H</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>s</td>
<td>seconds</td>
</tr>
<tr>
<td>SD</td>
<td>minimal synthetic defined media</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Sha</td>
<td>Shakdara</td>
</tr>
<tr>
<td>T</td>
<td>thymidine</td>
</tr>
<tr>
<td>T-DNA</td>
<td>transferred DNA</td>
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TF(s) transcription factor(s)
Tris 2-amino-2-hydroxymethyl-propane-1,3-diol
TT/tt TRANSPARENT TESTA
uidA β-glucuronidase
UTR untranslated region
UV ultraviolet
v/v volume per volume
Ws Wassilewskija
w/v weight per volume
w/w weight per weight
WT wild-type
x · g gravity force
X-gluc 5-bromo-4-chloro-3-indolyl-β-D-glucuronide
YPD yeast peptone dextrose medium

Nomenclature:

TT10 written in capital letters for the protein
TT10 written in capital letters, italics for the gene
tt10 written in small letters, italics for the mutant in the gene
pTT10:GUS transcriptional fusion of the TT10 promoter and reporter gene uidA
T1 primary transformants
T2 second generation of the T-DNA transformed plants

In the degenerated motifs, letters stand for nucleotides: N: A, C, G or T; R: A or G; W: A or T; Y: C or T
Chapter 1

Introduction

1.1 Seed biology

Formation of the seed is crucial in the life cycle of higher plants. Seeds serve several functions, among which the keys are nourishment of the embryo, dispersal to a new location, and dormancy to survive during unfavorable conditions. A typical seed of angiosperms, including *Arabidopsis* is constituted by the embryo enclosed in the endosperm, which supplies nutrients and a seed coat (integuments, testa) which provides protection (see Fig. 1.1 A.).

1.1.1 Seed coat development in *Arabidopsis*

In spermatophytes, the ovule is formed by the embryo sac which is surrounded by the nucellus and integument(s). After fertilization, the ovule develops into a seed containing a newly formed embryo immersed in the endosperm and enclosed in the testa. The seed coat is a maternal tissue derived from the differentiation of the integuments, whereas endosperm and embryo are of both maternal and paternal origin because of the double fertilization. The endosperm in *Arabidopsis* mature seed is reduced to one cell layer and is tightly associated with the seed coat.

The *Arabidopsis* testa includes two integuments in which micropyle pore is formed allowing entrance of the pollen tube, and the chalazal tissues. Three cell layers constitute the inner integument (ii), whereas the outer integument is two-layered. The five cell layers follow one of four distinct fates, e.g. subepidermal oil layer is accumulating flavonols, whereas the innermost cell layer (ii1, endothelium) specializes in proanthocyanidin (PA) biosynthesis. PAs are also accumulating in some cells in the chalazal region (pigment strand) creating a continuum of the tannin-producing cells. The vascular tissues of the maternal funiculus are connected with the seed in the chalazal region, which after seed detachment forms a scar called hilum. Formation of the testa involves cellular differentiation as well as programmed cell death. The cellular organization of the integuments is easily distinguishable at the heart stage of embryo development (see Fig. 1.1 A.), however further modifications are leading to the formation of the mature seed coat (see Fig. 1.1 B.). At the late stages of seed development, structure of the epidermal cells which were accumulating starch and forming mucilage is preserved. Other layers are crushed together by that time, PAs are released from the endothelial cells and impregnate the adjacent cell layers. The mature seed coat protects the embryo, contributes to seed dormancy by creation of a physicochemical barrier and helps seed dispersal. Many studies demonstrated that phenolic compounds, especially flavonoids play an important role in plant protection against environmental constraints (e.g. resistance to pathogens and herbivores, UV radiation) and also contribute to the germination-inhibiting
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Effects. Seed coat development has been recently reviewed in Haughn and Chaudhury (2005), and Debeaujon et al. (2007).

Figure 1.1: Testa structure and flavonoid localization in Arabidopsis seed. (A) Anatomy of a developing seed at the heart stage of embryo development (longitudinal section). Cells accumulating either proanthocyanidins or flavonols are highlighted in black or gray, respectively. The endothelium corresponds to the ii1 layer. (B) Cross section of a mature testa. c, chalaza; cl, columella; cpt, chalazal proliferating tissue (nucellus); ct, cuticle; cv, central vacuole; cw, cell wall; e, embryo; h, hyaline layer; ii, inner integument; m, micropyle; mu, muclilage; oi, outer integument; pe, peripheral endosperm (aleurone layer); ps, pigment strand; s, suspensor; vb, vascular bundle. Bar = 40 μm in A and 7 μm in B. (Adapted from Debeaujon et al., 2007).

1.2 Flavonoids in Arabidopsis seed

Flavonoids are plants secondary metabolites derived from the phenylpropanoid pathway. Most of the polyphenolic compounds, namely hydroxycinnamates, coumarins, lignins and flavonoids are derived from phenylalanine. Flavonoid structure is characterized by two aromatic cycles (A- and B- rings) linked by a heterocycle (C-ring) (see Fig. 1.2). At this moment there are more than 6500 molecules isolated and grouped in several classes according to the oxidation degree of the C-ring. These classes include flavonols, anthocyanins and flavan-3-ols. Additional modifications, including hydroxylations, methylations, glycosylations, acylations or prenylations account for the diversity within a compound class (Pourcel, 2006; Routaboul et al., 2006; Winkel-Shirley, 2006). The condensation of flavan-3-ols results in the formation of proanthocyanidins (Dixon et al., 2002; Marles et al., 2003; Pourcel et al., 2005).

Two classes of flavonoids, namely flavonols and proanthocyanidins are synthesized and accumulate in a tissue-specific manner in Arabidopsis seeds (Routaboul et al., 2006). Before ovule fertilization, cells of the endothelium layer are characterized by a dense cytoplasm. Rapid vacuolization occurs after fertilization and colorless tannin precursors can be detected (Devic et al., 1999). PA biosynthesis starts around 1-2 days after flowering (DAF). First, they accumulate in vacuoles of the endothelium cells as colorless compounds until 6-7 DAF (Debeaujon et al., 2003). At the beginning of seed desiccation (10 DAF) PAs stored in the vacuoles migrate to the cell walls, where oxidation occurs, leading to formation of brown pigments.
1.2. FLAVONOIDS IN ARABIDOPSIS SEED

contributing to the color of wild-type Arabidopsis seeds. Flavonols are found in the endosperm and embryo, however in the testa they are essentially accumulated in the oil layer. In mature seeds, flavonols are present mainly as glycoside derivatives with quercetin-3-O-rhamnoside being the most abundant (Pourcel et al., 2005; Routaboul et al., 2006). Interestingly, natural variation in the quantity of PAs and flavonols occurs among Arabidopsis accessions (Lepiniec et al., 2006; Routaboul et al., 2006), which might reflect an important trait of plant adaptation to different habitats. Flavonoid composition of mature arabidopsis seeds has been recently described in details by Routaboul et al., 2006.

![Figure 1.2: The structure of the flavonol quercetin.](image)

Quercetin is given as an example of carbon numbering. Important features influencing antioxidant potential are the di-hydroxylated B-ring, unsaturation and a 4-oxo function at the C-ring (see Williams et al., 2004). (Adapted from Pourcel et al., 2007).

1.2.1 Biosynthesis

A majority of the genes involved in flavonoid biosynthesis were identified through visual screenings of various collections of Arabidopsis mutants for altered seed coat pigmentation (Koornneef, 1990; Shirley et al., 1995; Lepiniec et al., 2006). Many mutants have been called transparent testa (glabra) for the altered pigmentation of the seed coat. One of the mutants, bangals (ban) is peculiar, because in place of PAs it accumulates anthocyanins. The transparent testa 10 (tt10) mutant is also unique in the fact that it does not affect the biosynthesis of PAs but their subsequent oxidative browning (Pourcel et al., 2005). It is important to note that six of the tt mutants are encoding transcription factors which are involved in the flavonoid biosynthetic genes regulation as well as they are required for normal seed development and cell differentiation. Flavonoid metabolism in seeds and mutants in genes affecting this metabolism have been recently reviewed in Lepiniec et al. (2006) and are summarized in Figure. 1.3.

The TT10/LAC15 gene encodes a polyphenol oxidase which belongs to a laccase multigene family of 17 members (Pourcel et al., 2005). TT10/LAC15 has been shown to be involved in the formation of epicatechin quinones that spontaneously polymerize into brown derivatives. Moreover, it may catalyze the oxidative browning of colorless PAs, which is consistent with the fact that the tt10 mutant accumulates wild-type PA levels but yellow seeds at harvest. TT10/LAC15 may also be involved in the formation of biflavonols from quercetin-3-O-rhamnoside (see Fig. 1.3 and Fig. 1.5). Interestingly, natural variation in TT10 transcript accumulation occurs between Arabidopsis accessions (Pourcel et al., 2005).
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Figure 1.3: Flavonoid biosynthesis pathway in Arabidopsis. Three major flavonoid end-products are formed: colorless proanthocyanidins (condensed tannins), which brownish upon oxidation by the TT10 / LAC15 laccase and are seed-coat specific, flavonol derivatives (colorless) which are ubiquitous and can be dimerized by TT10 to form biflavonols, and anthocyanins (purple), which are only in vegetative parts. Enzymes are presented in capital letters. Regulatory factors are in bold capital letters and circled. Corresponding mutants are in italics green. Abbreviations: ANR, anthocyanidin reductase; CE, condensing enzyme; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol reductase; F3H, flavonol 3-hydroxylase; F3’H, flavonol 3’-hydroxylase; FLS, flavonol synthase; GT, glycosyltransferase; LAC, laccase; LDOX, leucoanthocyanidin dioxygenase; OMT, O-methyltransferase; PFG, production of flavonol glycosides; RT, rhamnosyltransferase; tt, transparent testa; ttg, tt glabra. (Adapted from Lepiniec et al., 2006; Pourcel, 2006 and Stracke et al., 2007).
1.2. FLAVONOIDS IN ARABIDOPSIS SEED

1.2.2 Biological roles of flavonoids

Flavonoids are important in many aspects of plant life in relation to interactions with environment. It has been associated with resistance to pathogens and herbivores (Dixon and Paiva, 1995; Dixon et al., 2002). It seems that flavonoids can also contribute to seed longevity by providing protection against various biotic and abiotic stresses (Debeaujon et al., 2000; Winkel-Shirley, 2002; Rajjou et al., 2008). They can also reinforce testa-imposed dormancy by decreasing seed coat permeability (Debeaujon et al., 2000). Flavonols have been shown to act as signaling molecules for bacteria forming nitrogen-fixing nodules. They are also required for pollen tube germination (Taylor and Grotewold, 2005). Among other functions of flavonoids it is important to point their antioxidant and antimicrobial properties which are beneficial not only for plants but also for human and animal health (Scalbert et al., 2005; Selmi et al., 2006; Aron and Kennedy, 2008). However, some can also have a negative impact on the industrial use of some crop seeds (e.g rapeseed), or on protein digestibility and absorption from seed meal (Winkel-Shirley, 2001a; Marles et al., 2003; Dixon et al., 2005).

1.2.3 Oxidation of end-products

The protective function of flavonoids against biotic and abiotic stresses can be related to their cytotoxicity and antioxidant abilities. It seems that flavonoid oxidation contributes to these physiological and chemical properties. The oxidation of colorless flavan-3-ols and proanthocyanidins reinforces their cross-linking to the cell wall and leads to the formation of brown seed coat pigments, whereas oxidation of flavonols results in formation of antifungal agents. The topic of flavonoid oxidation in plants has been recently reviewed in Pourcel et al. (2007).

Flavonoid oxidation in planta is mainly mediated enzymatically by polyphenoloxidases (PPOs) and peroxidases, however it can also occur spontaneously (see Fig. 1.4 and Fig. 1.5). PPOs and peroxidases have been shown to be expressed during normal plant development, however they can be also induced by various environmental causes, like pathogen attacks, wounding or desiccation (Mayer and Staples, 2002; Thipyapong and Steffens, 1997; Thipyapong et al., 2004; Mayer, 2006). Physiological role of those genes seems to be regulated by differential subcellular localization of enzymes and substrates as well as by transcriptional and post-transcriptional events during gene expression.

Browning of Arabidopis seed coat is mainly due to the oxidation of epicatechin and soluble PAs by TT10 laccase (Pourcel et al., 2005). The tt10 mutant displays a delay in seed coat browning, an increase in soluble (non-oxidized) PAs and a default in the formation of biflavonols from quercetin-3-O-rhamnoside. The pattern of TT10 promoter activity analyzed with the GUS (see Fig. 1.6 A.) and GFP reporter genes as well as in planta TT10 activity (see Fig. 1.6 D.) showed that TT10 colocalizes with tannin-and flavonol-accumulating cell layers in the seed coat (see Fig. 1.6 andPourcel et al. (2005). Recent results confirmed that TT10 mRNA and also protein were localized in endothelium and outer integument 1 cell layer (see Fig. 1.6 B. and C.).
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Figure 1.4: Enzymatic reactions for polyphenoloxidases (laccase, catechol oxidase). Polyphenoloxidases (PPOs) are oxidoreductase enzymes acting on diphenols in presence of molecular oxygen ($O_2$). Laccase (LAC) is able to oxidize both ortho- and para-diphenols. Catechol oxidase (CO) is able to oxidize ortho-diphenols. (Adapted from Pourcel et al., 2007).

Figure 1.5: Seed coat pigmentation in Arabidopsis: illustration of a browning process. (a) Pictures showing the apparition of a brown pigmentation in the testa of the wild-type genotype (WT) during seed desiccation. The brown pigment is absent from the transparent testa 10 (tt10) mutant defective for a laccase enzyme. (b) Mutant tt10 seeds slowly get brown after harvest until resembling WT seeds. Bar = 550 μm. (c) Schematical drawing pointing to the occurrence of brown pigmentation during Arabidopsis seed development. daf, days after flowering. (Adapted from Pourcel et al., 2007).
1.2. FLAVONOIDS IN ARABIDOPSIS SEED

Figure 1.6: Seed coat sections showing the co-localization of TT10 promoter activity, mRNA expression and protein activity with flavonoid substrates (5-daf seeds).

(A) Promoter activity (pTT10:uidA); (B) In situ hybridization with TT10 cDNA probe; (C) Immuno-localization of TT10 protein with anti-HA antibody (pTT10:TT10:3xHA); (D) Protein oxidative browning activity in presence of epicatechin substrate; (E) Schematic representation of the integumentary structure showing flavonoid localization.

B. and C. are unpublished data from Lucille Pourcel. A., D. and E. are from Pourcel et al., 2005.

Abbreviations: em, embryo; ii, inner integument; oi, outer integument; PAs, proanthocyanidins; HA - hemagglutinin.

1.2.3.1 Laccases

Laccases (LAC), i.e. o-and p-diphenol:dioxygen oxidoreductases (EC 1.10.3.2) belong to a larger group of enzymes called multicopper or blue copper oxidases, which includes ascorbic acid oxidase and ceruloplasmin (reviewed in Mayer and Staples, 2002). These glycoproteins are characterized by four histidin-rich copper binding domains and ability to oxidize phenolic substrates in the presence of molecular oxygen (see Fig. 1.4). Also polyphenol oxidases of the catechol oxidase type (EC 1.10.3.1) require molecular oxygen for their activity, what makes them distinct from peroxidases, which require hydrogen peroxide (Pourcel et al., 2007).

Laccases have been found in eukaryotes (fungi, plants and insects) as well as in prokaryotes (Claus, 2004; Riva, 2006). The most studied are the fungal laccases, which are involved in spore pigmentation, virulence and delignification (Mayer and Staples, 2002; Pourcel et al., 2007). Plant laccases are less studied and until now were mainly associated with lignification, even if a direct activity of the enzyme on monolignol polymerization within the cell matrix has not yet been demonstrated (Mayer and Staples, 2002). Plant laccases were also shown to be involved in wound healing, iron metabolism detoxification and browning.
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1.2.3.2 Catechol oxidases

Catechol oxidases (CO) are enzymes catalyzing the oxidation of monophenols to o-diphenols as well as they can oxidize o-diphenols to the corresponding o-quinones (Marusek et al., 2006) (see Fig. 1.4). Like laccases, COs are glycosylated copper-binding proteins, that require molecular oxygen for their activity. They were found in eukaryotes and prokaryotes (Marusek et al., 2006; Mayer, 2006). Interestingly, the physiological roles of CO seem to be very close to those reported for laccases. It is not surprising as both groups of enzymes are involved in oxidative browning which in consequence can contribute to protection against UV radiations, and also senescence, wound healing and seed coat hardening (Pourcel et al., 2007 and references therein).

Interestingly, it seems that the Arabidopsis genome does not contain any typical catechol oxidase (Pourcel et al., 2005). Their lack could have been filled by laccases, in the sense of a specialization toward o-diphenol oxidation.

1.3 Regulation of gene expression

Regulation of gene expression includes all the processes leading to formation of the gene product, RNA and protein. All the steps of gene expression may be modulated, from gene transcription to the post-translational modifications of a protein. However, expression of many genes is regulated at the transcriptional level and depends on the combinatorial contribution of transcription factors, which can act as activators or repressors. Gene expression regulated at the transcriptional level results in precise spatio-temporal manner activity of their promoter. Below, expression patterns of flavonoid-related genes are given as an example.

1.3.1 Regulation of BAN and MYBL2 expression

Regulation of BAN (Debeaujon et al., 2003) and MYBL2 (Dubos et al., 2008) differs at the level of the spatial activities of their promoter in the seed coat: BAN is present in one integumentary cell layer (ii1) and MYBL2 is present in two cell layers (ii1 and oi1).

The entry step into the proanthocyanidin branch of flavonoid biosynthesis is formation of epi-flavan-3-ols. This reaction is metabolized by anthocyanidin reductase encoded by BANYULS (BAN) (see Fig. 1.3). BAN promoter activity was detected specifically in PA-accumulating cells in the the inner integument 1 layer (endothelium) of the seed coat and pigment strand in the chalaza zone (see Fig. 1.7 G. and Debeaujon et al. (2003). The BAN promoter deletion and gain-of-function experiments led to identification of 86-bp promoter region which functions as an enhancer specific for PA-accumulating cells. Studies of the BAN promoter activity in regulatory mutant background revealed that its activity was abolished in tt2, tt8 and ttg1 (Debeaujon et al., 2003). The spatial pattern of BAN promoter activity was also modified in tt1 and tt16, but not in tlg2 (Debeaujon et al., 2003). Further studies revealed that TT2/TT8/T TG1 regulatory complex binding to the enhancer element in BAN promoter is required for its expression (Baudry et al., 2004).

Recently, a MYB family transcription factor, MYBL2 has been shown to regulate the transcription of genes involved in anthocyanin biosynthesis (Dubos et al., 2008). Although MYBL2 promoter activity is detected in vegetative tissues, in seeds its activity is restricted to the endothelium and epidermis (see Fig. 1.7 H. and Dubos et al., 2008). Cis-acting regulatory elements in promoter region and transcription factors required for MYBL2 expression are yet unknown.
1.3.2 Two-cell layer expression pattern of \textit{TT10}

\textit{TT10} promoter activity colocalizes first with PA-and flavonol-producing cells of the testa. Activity of \textit{TT10} promoter starts at early stages of seed development. First it is detected in the endothelium and in the pigment strand at the chalaza zone (see Fig. 1.7 A., C., and D.). Later, the activity increases and spreads to the outer integument 1 cell layer of the seed coat (see Fig. 1.7 E., and F.). The \textit{uidA} gene was also strongly expressed in early aborted seeds (see Fig. 1.7 B.). Any difference in GUS activity compared with the wild type was observed in three regulatory mutants \textit{tt2}, \textit{tt8}, and \textit{ttg1} (Pourcel et al., 2005).

1.3.3 One layer, two layers, which layers?

It seems that regulation of gene expression in the seed coat is very complex. Genes can be expressed in one or more layers of the testa. Moreover they can be expressed at various developmental stages of the seed coat development. Identification of \textit{cis}-acting regulatory elements required for gene expression in each of the seed coat cell layers or combination of layers would allow to target expression of other genes for crop improvement.

1.4 Objectives of the thesis

The main focus of this thesis was the functional analysis of the \textit{TRANSPARENT TESTA 10} gene. The work was following two axis, where developmental and possible stress regulation of \textit{TT10} expression was studied.

The first part of the work dealing with the developmental regulation of \textit{TT10} expression required detailed characterization of promoter activity throughout the plant to complete existing data. Functional analysis of \textit{TT10} promoter was aiming at identification of the promoter region and \textit{cis}-acting regulatory elements required for seed coat specific activity. Identification of the CAREs allowing to drive gene expression separately in endothelium (PA specific) or outer integument 1 (flavonol specific) cell layer, were one of the goals.

In parallel, existing natural variation for \textit{TT10} expression was exploited as it could provide an additional information about regulation of gene expression.

Several complementary approaches, namely studies of \textit{TT10} promoter activity and gene expression level in candidate regulatory mutant background as well as transcription factor library screening were aiming at identification of transcription factors which could directly regulate \textit{TT10} expression.

Second part of the work dealing with possible stress regulation was aiming at identification of the stress conditions in which \textit{TT10} would be expressed. The tools developed in the first part were planned to be used for identification of the promoter region and \textit{cis}-acting regulatory elements involved in the stress regulation of \textit{TT10} expression.

The secondary objectives of this thesis were aiming at identification of other laccases expressed in seeds and manipulations of \textit{Arabidopsis} cell suspensions to study flavonoid metabolism in simplified system.
Figure 1.7: Spatial pattern of *TT10* promoter activity in WT seeds. Comparison with *BAN* and *MYBL2* promoter activities.

(A) to (F) Expression of the Pro*TT10*:uidA cassette in developing seeds at 1 DAF A, 3 DAF C and D, 8 DAF E and F, and in an aborted seed B. GUS activity is observed with Nomarski optics on whole mounts for A to C and F, and on sections for D and E.

(G) Pattern of *pBAN* activity in developing seed at 4 DAF.

(H) Pattern of *pMYBL2* activity in developing seed at 4 DAF.

Abbreviations: c, chalaza; em, embryo; e, endothelium; ii, inner integument; m, micropyle; oi, outer integument. Bars = 24 μm for F and K; 70 μm for A to D, G and H.

Adapted from Pourcel et al., 2005: A to F; Debeaujon et al., 2003: G and Dubos et al., 2008: H.
Chapter 2

Results

The main objective of my work was to study the regulation of \textit{TT10} gene expression. The results of experiments, techniques and approaches, concerning different aspects of gene expression, which have been used to shed light on the mechanism of \textit{TT10} expression, are described in details in following sections: \textit{TT10} expression and promoter studies (section: 2.1), techniques aiming at finding transcription factors involved (section: 2.2), insights from natural variation occurring for \textit{TT10} expression (section: 2.3) and potential stress response (section: 2.4). Each section consists of a short introduction, presentation of the results and technical discussion of the approach used.

Two secondary objectives of this work were: I) to identify other laccases expressed in seeds (section: 2.5) and II) to establish conditions and optimize techniques for the manipulation of \textit{Arabidopsis} cell suspensions as a tool to study the flavonoid metabolism (section: 2.6).

2.1 Transcriptional regulation of \textit{TT10} expression

This section is focusing on \textit{TT10} expression during plant development and detailed characterization of \textit{TT10} promoter. At first, our analysis of \textit{TT10} transcript accumulation is presented and compared to publicly available expression data (2.1.1). Then the promoter activity is studied during plant development, using \textit{uidA} reporter gene (2.1.3). \textit{In silico} analysis (2.1.2) of the promoter sequence was used as a basis to design the 5’ dissection of the promoter (2.1.3.1), and its site-directed mutagenesis (2.1.3.4). \textit{In silico} analysis of the promoter sequence is supported by literature search, documenting on the functionality of \textit{cis}-acting regulatory elements (CARE).

2.1.1 \textit{TT10} transcript accumulation during plant development

2.1.1.1 Transcript accumulation - RT-PCR

The expression pattern of \textit{TT10} was previously investigated by RT-PCR (McCaig et al., 2005; Cai et al., 2006) and semiquantitative RT-PCR (Pourcel et al., 2005). In those studies \textit{TT10} transcript was detected to be: i) in all the plant organs except stem (McCaig et al., 2005), ii) predominantly in developing silique, at low level in stem, seedling and flower but not in root (Pourcel et al., 2005), iii) in silique, root, flower and stem but not in leaf (Cai et al., 2006). Here, we wanted to revisit and complete previously known and sometimes contradictory data using the very sensitive quantitative RT-PCR method, with special attention to silique development. More robust and precise data about \textit{TT10} expression were required for the analysis of its regulation. Quantitative RT-PCR had also been chosen to study differences in \textit{TT10} transcript
accumulation between accessions (see: 2.3.1) as well as in candidate regulatory mutant backgrounds (see: 2.2.3, 2.2.4 and 2.2.5).

The expression pattern was investigated in various tissues of 6-week-old Col-0 plants grown in long day conditions in the greenhouse (Fig. 2.1 A.). The TT10 mRNA was detectable as early as the open-flower stage. Detailed analysis of transcript accumulation in developing siliques, revealed a huge increase between 5 and 7 days after flowering (DAF) followed by a slight decrease at 10 DAF, and a second peak of transcript accumulation around 15 DAF. In older siliques the amount of TT10 mRNA decreases and is close to 0% of ELONGATION FACTOR 1a4 (EF1) control at 21 DAF, but is still detectable. TT10 is highly expressed in developing seeds, reaching up to 350 %EF at 5 DAF and 250 %EF at 10 DAF (Fig. 2.1 B.). TT10 transcript was not detected in dry seeds, cauline and rosette leaves, stem at the lower part and terminal node.

![Figure 2.1: Expression pattern of the TT10 gene in developing siliques and seeds of wild-type Col-0 plants.](image)

TT10 gene expression in wild-type Col-0 plants was detected by quantitative RT-PCR and is presented as a percentage of the expression of the reference gene ELONGATION FACTOR 1a4 (EF1). For details see 3.2.5. (A) Picture illustrates siliques development and samples used for RNA extraction. Graph is representing one out of two biological repeats of the experiment. (B) TT10 gene expression in extracted seeds. Values represent averages ±SE of three technical replicates. Bar = 2 cm.

### 2.1.1.2 Transcript accumulation - Transcriptomics

Publicly available transcriptomic data are a constantly growing resource, therefore we checked (last update 7.04.2009), if new combinations of tissue/developmental stage/treatment have revealed new information about TT10 expression. Genevestigator, Bio-Array Resource (BAR), Gene Co-expression Analysis Toolbox (GeneCAT), DIURNAL and several other analysis and visualization tools were used (see section 3.2.9). For many functions those web-based tools are redundant, but for some analysis one is more adapted than the other. Below (Fig. 2.2) as an example, analysis of TT10 expression with the eFP Browser (at BAR) is presented. This tool is well adapted to gene expression analysis thanks to easily readable color code and graphic representation of plant organs/tissues for which the data are presented. Fast switch between data sets (e.g. developmental/stress), standard deviation and threshold filtering as well as easily accessible expression...
2.1. TRANSCRIPTIOINAL REGULATION OF TT10 EXPRESSION

values for more detailed analysis are making eFP Browser a very convenient tool for gene expression studies. The TT10 expression analyzed and visualized with the eFP Browser confirms that it is mainly expressed in developing seed/silique but also shows expression in pollen grain (Fig. 2.2).

- This probe set reaches its maximum expression level (expression potential) of 3258.06 in the Developmental Map or Tissue:

![Heat map of TT10 expression](image)

**Figure 2.2: TT10 expression visualized with eFP Browser.**

eFP Browser is presenting gene expression as a heat map placed on the graphic representation of the Arabidopsis organs - Developmental Map - AtGenExpress Developmental Set (Schmid et al., 2005). Standard deviation filtering has been used to mask samples with the deviation greater than half of their expression value (grey). Signal threshold filtering has been set at 100 (expression potential) to eliminate samples where expression is considered to be below the detection level (yellow). Data are not available for separate siliques (white) from torpedo stage of embryo development onwards, where only dissected seeds were analyzed. Developing seed, silique and pollen where TT10 gene expression potential is > 100 (detectable) are painted in red. For details see: [http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi](http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) and Winter et al. (2007).

Figure 2.3 is presenting comparison of TT10 and BAN expression, which is nicely visualized thanks to Expression Profiling Tool available at GeneCAT. This tool allows comparing the expression of multiple genes in different plant tissues. BAN is a core enzyme of the subpathway of flavonoid biosynthesis leading to proanthocyanidins, which are later oxidized by TT10 (Debeaujon et al., 2003; Pourcel et al., 2005). The GeneCAT analysis shows two peaks of TT10 expression in developing seeds/siliques as well as weak signal in pollen and even weaker in flower and root. From the GeneCAT analysis BAN expression seems to be seed/silique specific, with a peak of expression at an earlier stages than TT10, what corresponds to the RT-PCR results presented in Debeaujon et al. (2003).
2.1.1.3 Analysis of publicly available transcriptomic data for TT10 expression during plant development - Summary

Taken together all the transcriptomic data analyzed, it seems that TT10 accumulation is significant only in developing silique and seed. In some experiments, TT10 is on limit of detection level (pollen and root), however there are limited repetitions available and the standard variation is high for those data which should therefore be treated with caution. TT10 expression is below the detection level in the circadian/photoperiod experiments.
2.1. TRANSCRIPTIONAL REGULATION OF TT10 EXPRESSION

Figure 2.4: Comparison of TT10 and CHS expression visualized with DIURNAL. TT10 and CHS expression in long and short day is presented as a graphical output adapted from the DIURNAL gene expression analysis tool. TT10, closed green and open blue squares for long and short days respectively. CHS, closed red and open orange diamond/rhombus for long and short days respectively. Samples, 7-day-old seedlings were grown in MS agar medium supplemented with 3% sucrose. For details see: http://diurnal.cgrb.oregonstate.edu/diurnal_details.html and Mockler et al. (2007).

2.1.2 In silico analysis of TT10 promoter sequence

Several web-based computer programs (see 3.2.9) recognizing known Cis-Acting Regulatory Elements (CAREs) have been used for the in silico analysis of the 2.0-kb TT10 promoter sequence Col-0, including the 5’ UTR (for promoter sequence used in the study see Appendix, 5.1). Detection of CAREs in the promoter by so-called ’search by signal’ methods are prone to give false positive results (Rombauts et al., 2003). For that reason the results obtained should be treated as putative cis-acting regulatory elements and their functionality should be tested in vivo. In order to select the best candidates for further analysis, literature mining accompanied in silico analysis to find out the context and organization of the motifs in the promoter.

PLACE SignalScan server was the preferred tool as it is searching for the presence of motifs identical or similar to the previously reported cis-acting regulatory elements in the PLACE database, which presently seems to be the richest with 469 entries of CAREs deposited (PLACE 30.0, 469 entries, Jan.8, 2007). The full list of putative cis-acting regulatory elements found in the TT10 promoter is presented in the Appendix, Table 5.1. Similar results were obtained when querying PlantCARE, AtcisDB at Agris (see Appendix Tab. 5.2), ATHENA and PlantPan. In addition, in silico analysis revealed no tandem repeats regions, nor CpNpG islands in -1000bp - + 500bp of the At5g48100 locus (PlantPan) and no over-represented TF binding site has been found (ATHENA).
It is important to note that *TT10* is not sharing its promoter sequence with any known or predicted gene and that the preceding gene (At5g48110) is transcribed from the same strand. The region considered as *TT10* promoter is the 3' region for that gene (Fig. 2.5). In such a case any changes arising in the promoter region on the course of the evolution would probably affect essentially *TT10* expression.

Arabidopsis thaliana Chromosome 5

The organization of the *TT10* genomic region on chromosome 5 (top, blue rectangle) is presented. Transcription units are represented with arrows indicating the orientation of the transcripts. Bottom part is showing the gene structure where rectangles are representing exons and lines introns. UTR’s, when present are shaded orange. Figure adapted from ATIDB.

2.1.2.1 Putative *cis*-acting regulatory elements found in the *TT10* promoter

Studies on yeast promoters suggest that regulatory elements are commonly present in the 500bp upstream region of the transcription initiation site (Caselle et al., 2002). Many plant genes have been shown to contain regulatory elements required for the tissue specific gene expression in the proximal part of their promoters (Abe et al., 2003; Debeaujon et al., 2003; Nakashima et al., 2006; Zhou et al., 2009). For that reason special attention has been taken to analyze this part of the *TT10* promoter in details as well as to focus on the dissection of the proximal part of the promoter (see 2.1.3.1). Selected putative *cis*-regulatory elements found in that part of the promoter are presented in Figure 2.6 and described below. Several criterions have been used to limit the list of putative CAREs considered: 1.) tandem repeats and length of the motif (longer regarded as more significant), 2.) literature evidence for being involved in seed development and/or defense/stress response (associated with putative function of laccases and flavonoids), and/or 3.) motifs similar to the one previously reported to be involved in flavonoid biosynthetic genes regulation.

Motifs are briefly described, moreover examples of transcription factors are given and briefly discussed in the context of the putative *cis*-acting regulatory elements.
2.1. TRANSCRIPTIONAL REGULATION OF TT10 EXPRESSION

2.1.2 Putative CAREs associated with seed development, stress response and/or protection against various biotic and abiotic stresses

In *Arabidopsis* seeds, flavonoids are accumulated and they are thought to provide protection against various environmental challenges and ensure embryo survival and seed germinability (Rajjou and Debeaujon, 2008). In particular, flavonoid oxidation triggered by *TT10* could be required for protection of the embryo against environmental challenges (Pourcel et al., 2005, 2007). Functions associated to laccases in plants are multiple. Apart from lignification, they are also thought to be involved in wound healing, plant defense against predators, herbivores and invasion by bacteria and fungi (Mayer and Staples, 2002). Plant hormones e.g. ABA, GA, ethylene, salicylic and jasmonic acids (SA and JA respectively) are involved not only in stress-induced gene expression, but also in plant and seed development and in cross-talk between those processes (Fujita et al., 2006; Chung et al., 2008; Pauwels et al., 2008, 2009; Santner et al., 2009). Taking in account that *TT10* encodes a laccase which is involved in flavonoid oxidation, *cis*-acting regulatory elements could be common to genes involved in both pathways and/or have multiple functions, i.e. motifs found in ABA and dehydration responsive genes and motifs in genes involved in seed development where their expression is mediated by ABA (Simpson et al., 2003; Borisjuk et al., 2004; Nakashima et al., 2006; Cao et al., 2007; Chinnusamy et al., 2008).

The putative CAREs for *TT10* regulation listed below were previously reported to be involved either in seed development or stress response.

- **W-box** - is a binding motif for WRKY transcription factors with the core sequence (T)TGAC(Y). Most of the studied WRKY TF appear to have a binding preference for the W-box, although the specificity is partly dependent on the sequence flanking the core motif (Ciolkowski et al., 2008). This group of TF seems to be unique to plants and it is involved in various physiological responses including pathogen defense, senescence and development (Eulgem et al., 2000). Recently WRKY TF have been also found in the protist *Giardia lamblia* and the slime mold *Dictyostelium discoideum*, which could indicate their earlier origin (Ulker and Somssich, 2004; Pandey and Somssich, 2009). It seems that they can act both as transcription activators and repressors. In rice, WRKY transcription factors have been shown to be transcriptional repressors of the gibberellin signaling pathway (Zhang et al., 2004), positive and negative regulators of ABA signaling (Xie et al., 2005), whereas in *Arabidopsis* they have been shown to be involved in salicylic acid signaling and NONEXPRESSER OF PR GENES 1 (NPR1) gene expression (Yu et al., 2001). W-boxes are present in promoters of many pathogen-related genes, and some of them are having as many as eleven W-boxes (Chen and Chen, 2002; Eulgem and Somssich, 2007). In the *TT10* promoter, several W-boxes have been found, but as the sequence outside of the core motif is also important, it is difficult to conclude about their functionality. It should be noted that one of the *TT* genes namely *TTG2*, is a WRKY transcription factor (AtWRKY44) involved in seed coat development and trichome formation in *Arabidopsis* (Johnson et al., 2002). It would be interesting to test if *TT10* could be a direct target of TTG2. Therefore we have tested the expression of *TT10* in *ttg2* mutant background (see section 2.2.2).

- **ABRE** (ABA Responsive Element) and **ABRE-like** - there are several overlapping motifs with the core ACCTG sequence. They are binding sites for basic leucine zipper (bZIP)-type binding proteins, which are very often transcriptional activators (Foster et al., 1994). In rice, the bZIP transcriptional activator *RITA-1* is highly expressed during seed development (Izawa et al., 1994). ABRE elements act together with dehydration-responsive elements in the *RD29A* gene expression in response to ABA (Nakashima et al., 2006). ABRELATERD1 - ABRE-like (motif: ACCTG) and ACUTATERD1 (motif: ACCTG) motifs are required for etiolation-induced expression of *ERD1* gene (Early Response to Dehyd-
CHAPTER 2. RESULTS

ration1) (Simpson et al., 2003). The ABRE motifs in the TT10 promoter are considered as important, because TT10 expression could be regulated by ABA during seed development. Indeed when at the last stages of development the seed is desiccating, this could resemble the response to dehydration.

**TCA1MOTIF** - (motif: TCATCTTCTT) - TCA-1 (Tobacco Nuclear Protein-1) binding site is related to salicylic acid-inducible expression of many genes. It has been found in more than 30 different plant genes which are known to be stress-induced. It was reported as a functional motif in barley, when repeated four times within 200bp (Goldsbrough et al., 1993). In TT10 promoter it is present only once in the 2.0-kb promoter analyzed, but as it is 10bp long motif, it could be meaningful.

**Laccase-related motifs:**

- **MYB** - Recently it has been shown that AtMYB58 and AtMYB63 are regulating expression of genes involved in lignin biosynthesis by binding to the AC element in their promoter (AC-I: ACCTACC, AC-II: ACCAACC, AC-III: ACCTAAC). Moreover it has been demonstrated that they can directly upregulate the expression of AtLAC4 but not AtLAC17 (Zhou et al., 2009). It seems that the MYB family TF and therefore MYB binding motifs are highly important elements for gene expression in secondary metabolism. Perfect AC elements were not found in TT10 promoter sequence, although in proximal 500bp, AC-I is present once with two mismatches, AC-II - once with one mismatch and AC-III twice with one mismatch.

2.1.2.3 CAREs found in promoters of flavonoid biosynthetic genes

Several classes/families of transcription factors (TF) have been demonstrated to be involved in the regulation of the flavonoid biosynthetic genes, among them are MYB TF: AtMYB11, AtMYB12, AtMYB111 (flavonols; Stracke et al. (2007), AtMYB75/PAP1, AtMYB90/PAP2, AtMYB113, AtMYB114 (Anthocyanins; Borevitz et al. (2000)), AtMYB123/TT2 (Proanthocyanidins; Nesi et al. (2001) and the negative regulators AtMYB4 (Jin et al., 2000) and AtMYB2 (Dubos et al., 2008). Other classes are represented by the bHLH family, with AtbHLH001/GL3, AtbHLH002/EGL3 (anthocyanins, Zhang et al. (2003) and AtbHLH042/TT8 (anthocyanins and proanthocyanidins; Nesi et al. (2000); Zhang et al. (2003). These bHLH factors are forming protein complexes with already mentioned AtMYBs involved in anthocyanin and proanthocyanidins biosynthesis, and the WDR regulatory protein TTG1 (Walker et al., 1999; Baud et al., 2004). The involvement of the other transcription factors AtWRKY44/TTG2 (Johnson et al., 2002), WIP-type Zn-Finger/TT1 (Sagasser et al., 2002) and MADS AtAGL32 - TT16 (Nesi et al., 2002) is less studied and the direct targets are not yet identified for them (reviewed in Lepiniec et al., 2006).

The motifs found in the TT10 promoter which could bind transcription factors of the families mentioned are:

- **MYB2** - motif recognized by Signal Scan as MYB1AT (binding motif: WAACCA). MYB recognition site in the promoters of dehydration responsive genes. Originally discovered in the promoter of the rd22 gene, which is up-regulated in the AtMYB2 / AtMYC2 (see below MYC2) over expressing plants in response to abscisic acid (ABA) under drought stress (Abe et al., 2003).

- **MYC2** - recognized by Signal Scan as MYCATORD22 (motif: CACATG) and MYCCONSENSUSAT (motif: CANNTG). Also described as E-box (binding site: CANNTG) and sometimes overlapping with ABRE (Stalberg et al., 1996). MYC2 is a binding motif for the AtMYC2/rd22BP1 TF belonging to bHLH family (Solano et al., 1995; Abe et al., 1997; Busk and Pagès, 1998). Involvement in cold stress response has also been reported (Chinnusamy et al., 2003).
Those two CAREs were reported to act together in many ABA and dehydration responsive genes (Abe et al., 2003). MYB-bHLH complex had been reported to be involved in the light-regulated activation of the phenylpropanoid genes (Hartmann et al., 2005). MYB2 and MYC2 sites in TT10 promoter are located approximately at -400bp and -250bp, positions which were found in many genes upregulated in plants overexpressing AtMYC2/AtMYB2 (Abe et al., 2003). It would be interesting to evaluate their functionality.

Several other MYB and MYB-like motifs have been found:

- **GARE MYB** - MYBBAHV (motif: TAACAAA). This motif is an element of the gibberellin response complex of the alpha-amylase gene in barley (Gubler et al., 1995). GARE MYB is overlapping with AMYBOX1 (motif: TAAACARA), which is a conserved sequence in the promoters of the alpha-amylase gene of barley (Hordeum vulgare), rice (Oryza sativa) and wheat (Triticum aestivum) (Huang et al., 1990). This motif is also similar to GAREAT (motif: TAACAR) which has been reported as gibberellin(GA)-responsive element present in 20% of GA inducible genes during seed germination (Ogawa et al., 2003).

- **MYB** - MYBCOREATCYCB1 (motif: AACGG). Motif found in promoter of the CycB1;1 gene, which is involved in cell cycle regulation. It had been found to be necessary for the binding of the proteins (e.g. putative MYB TF, At2g03470) and able to drive GUS expression in plants overexpressing hypothetical protein, At2g13640 (Planchais et al., 2002).

2.1.2.4 Additional comments about known transcription factors involved in flavonoid biosynthesis

- Pourcel et al. (2005) have shown that AtMYB123/TT2, AtbHLH042/TT8 and WDR/TTG1 regulatory protein, are not required for the activity of the 2.0-kb TT10 promoter in transgenic plants.

- If the situation is clear for above mentioned TT2, TT8 and TTG1 transcription regulators, it seems to be much more complex for WIP-type Zn-Finger/TT1, MADS AtAGL32/TT16 and AtWRKY44/TTG2. Those genes are not only involved in flavonoid biosynthesis but also in endothelium development. It is in the scope of this study to investigate the involvement of TT1, TT16 and TTG2 in TT10 expression (discussed in more detail in section 2.2.1; see also: W-box above).

2.1.2.5 Other CAREs

Motifs of lower abundance in TT10 promoter, shorter binding site or which could be indirectly associated with TT10 gene function are listed here.

- **DOF** - (motif: AAAG) - is a short motif binding zinc finger for plant specific DOF TF, which are associated with expression of multiple genes involved in carbon metabolism (Yanagisawa, 2000). There are several DOF motifs in proximal part of the TT10 promoter and surprisingly, most of them (five) are spanned within 150bp region.

- **ARR1** - (motif: NGATT) - ARR1-binding element is a motif recognized by ARR1 and ARR2 response regulator proteins which faintly resemble mammalian MYB TF (Sakai et al., 2000).

- **ACTCAT** - motif recognized by Signal Scan as PREATPRODH (motif: ACTCAT), which stands for Pro- or hypoosmolarity element. ACTCAT element has been show to bind several bZIP class TF and is necessary for the efficient expression of proline dehydrogenase gene in response to L-Proline and hypoosmolarity (Satoh et al., 2002, 2004; Weltmeier et al., 2006).
• **I-box** - motif recognized by Signal Scan as IBOXCORE (motif: GATAA). This motif is a conserved sequence in promoters of light-regulated genes in both monocots and dicots (Terzaghi and Cashmore, 2003).

• **-10 PE** - recognized by the Signal Scan as -10PEHVPSBD (binding motif: TATTCT). Motif originally found in barley as involved in light regulation of chloroplast *psbD* gene (Thum et al., 2001).

• **SORLREP3AT** - (motif: TGTATATAT) is one of the computationally identified Sequences Over-Represented in Light-Repressed Promoters (SORLREP) in *Arabidopsis* (Hudson and Quail, 2003).

Considering the protective role of flavonoids against UV radiation (Winkel-Shirley, 2002) and reported involvement of light in regulation of flavonoid biosynthetic genes (Thain et al., 2002), I-box, -10PE and SORLREP3AT were selected as candidate motifs.

**Seed related CAREs:**

• **E-box** - EBOXBNNAPA (motif: CANNTG). Motif is overlapping with MYC and ABRE. E-box has been shown as required for high expression of napA storage protein in transgenic *Brassica napus* seeds (Stalberg et al., 1996).

• **DPBF** - (motif: ACACNNG) - Element found by yeast one hybrid screen in a carrot Dc3, lea class gene (Kim et al., 1997). Motif is for binding of DPBF (Dc3 Promoter-Binding Factor, bZIP class TF), which shares up to 96% sequence similarity with ABI5 in the DNA binding and potential dimerization domains (Finkelstein and Lynch, 2000).

**2.1.2.6 TATA box**

Predicted TATA box is in the 42bp distance form the beginning of the 5’ UTR identified by RACE PCR (Pourcel et al., 2005). Interestingly at the beginning of the transcript, W-box and GT1CONSENSUS (GT-1) sequences co-localize. GT-1 binding site is present in many promoters of light-regulated genes, where it can stabilize the TFIIA-TBP-DNA (TATA box) complex (Gourrierec et al., 1999). Moreover binding of the GT-1 like factors to the *PATHOGENESIS-RELATED GENE 1a* (*PR-1a*) promoter influences the level of SA-inducible gene expression (Buchel et al., 1999). We could hypothesize that W-box could be part of a negative regulation, whereas GT-1 would act for activation.

**2.1.2.7 In silico analysis of TT10 promoter sequence - Summary**

The *in silico* analysis of the *TT10* promoter sequence, accompanied by literature mining is a base for the understanding of gene regulation. To sum up the information presented in this section, several hypotheses could be proposed. If *TT10* is regulated as one of the flavonoid biosynthetic genes, there is probability that MYC2 and MYB2 motifs are functional and that they are binding sites for bHLH and MYB transcription factors respectively. Second hypothesis is built on the assumption that *TT10* could be regulated as a pathogen related gene. In this case we could also expect that the MYB sites would be involved and perhaps accompanied by W-boxes and/or ABRE. Another hypothesis is that *TT10* is regulated in a completely novel manner. It is possible, because it is not involved directly in flavonoid biosynthesis (like early and late biosynthetic genes), but in oxidative modification of end products. The 5’ promoter dissection experiment described in the next section was based on the *in silico* information gathered. CAREs with the functional evidences were separated in various promoter constructs to validate which ones are functional *in vivo*. 
2.1. TRANSSCRIPTIONAL REGULATION OF TT10 EXPRESSION

A.

Fig. In Silico analysis of cis-regulatory motifs in AtTT10 promoter. In bold are motifs considered for site directed mutagenesis (details of the modifications). Nucleotides modified in the constructs are in bold uppercase and the resulting sequences are named (A)

B. Wild-type Col-0 nucleotide sequence and selected putative cis-regulatory DNA elements found in TT10 promoter. In bold are motifs considered for site directed mutagenesis (details of the modifications). Solid arrows are covering the corresponding motifs indicating their orientation. Dashed arrows are representing primers and cover the 'binding region'. 5' UTR is underlined. Vertical arrow is pointing nucleotide substitution before ATG, which are due to creation of NcoI restriction site required for transcriptional fusion with the reporter gene.

(B) Wild-type sequence of the putative cis-regulatory element analyzed in site-directed mutagenesis experiment. Nucleotides modified in the constructs are in bold uppercase and the resulting sequences are named M1 to M5.

Figure 2.6: In silico analysis of proximal (500bp) TT10 promoter showing putative cis-acting regulatory DNA elements.
2.1.3 Studies of TT10 promoter activity during plant development

Pourcel et al. (2005) have investigated the regulation of TT10 at the transcriptional level with a 2.0-kb TT10 promoter from Columbia (Col-0) translationally fused to the uidA reporter gene encoding β-glucuronidase (GUS) protein transferred in Ws-4 background (previously named Ws-2). The promoter activity at the early stages of embryo morphogenesis (1 - 3 DAF) was detected in the endothelium layer of the seed coat and in the pigment strand at the chalaza zone. Later, the activity increased and was also observed in the outer integument (mostly in the oil layer of the seed coat). The GUS activity was also reported in early aborted seeds and in the transmitting tissue of the siliquer. Similar results were reported by Liang et al. (2006a), where activity of 1.5-kb TT10 promoter activity was studied.

The present study intended to analyze the activity of the TT10 promoter in its native background and extend it to the whole plant as well as to develop a tool which could be used for the analysis of stress response (see: 2.4).

2.1.3.1 5' dissection of the TT10 promoter

To investigate further the regulation of TT10 and to define the domains of the promoter that might be important for the transcriptional regulation of tissue specificity, 5'-deletions were generated and translational fusions to the uidA gene were made.

Promoter fragments studied and cloning strategy

To limit the number of constructs and to maximize the output of the information about the promoter region which might be important, in silico analysis and putative CAREs were considered to plan promoter dissection, focusing on the proximal part of the promoter (as described in section 2.1.2). The promoter fragments studied were numbered, where: 1. stands for the shortest fragment of 173bp, 2. 271bp, 3. 465bp, 4. 1.0-kb, 5. 1.5-kb and 6. for 2.0-kb. Constructs were named as pTT10_X:GUS/pBIB-Hyg, where X stands for the promoter fragment considered.

The shortest (1.) promoter fragment includes a predicted TATA box located 42bp upstream from the 5'UTR and putative CAREs: ABRE, MYB and TCA1MOTIF. Next fragment (2.) was planned to include two W-boxes which could be functional together. Fragment 3., includes MYC2, MYB2, ABRE and several other putative CAREs (see Fig. 2.6). Two other constructs have been planned to divide the upstream remaining sequence in equal parts, resulting in fragments 4. and 5.

The cloning strategy was aiming to conserve the native promoter composition (only two nucleotides changed to create Ncol restriction site) and is based on cloning of 2.0-kb TT10 promoter described in Pourcel et al. (2005). Briefly, promoter fragments were PCR-amplified with the primers (Appendix, Tab. 5.3) including XhoI and NcoI restriction sites for translational fusion with the uidA (GUS) gene and sub-cloned into pCR®-Blunt II-Topo®. The XhoI-NcoI digested fragments were cloned into the pBS-GUS vector (Debeaujon et al., 2003), which was subsequently digested Smal-KpnI to recover the corresponding promoter:GUS cassettes. The introduction of the constructs into the pBIB-Hyg binary vector was a limiting step of the cloning and it appeared to be of very low efficiency. It is important to note that the vector had to be prepared in two-step digestion, first with Smal and then KpnI, because those sites are overlapping and reverse order of digestion results in loss of the Smal site. Constructs were verified by restriction and sequencing. To fully appreciate the tissue specificity of the promoter, Arabidopsis Col-0 and Ws-4 wild-type plants were transformed with the six constructs described above. The results are illustrated on Figure 2.7, described below and summarized in table 2.1.
2.1. TRANSCRIPTIONAL REGULATION OF TT10 EXPRESSION

2.1.3.2 In planta analysis of GUS activity

The consistent GUS pattern established for each of the promoter fragments studied is based on analysis of 24 independent transformants lines (T1) (except pTT10_1:GUS/pBiB-Hyg, 12 lines were studied). All the transgenic plants studied were genotyped for each construct and T2 plants were hygromycin selected to chose those segregating 3:1. Characteristic patterns of each promoter fragment activity observed in stable transformants lines (T2 and T3 generations; hygromycin resistant) was consistent with the one observed in primary transformants.

The same spatio-temporal GUS activity patterns were observed for pTT10_3, pTT10_4, pTT10_5 and pTT10_6 promoter fragments (Fig. 2.7). For those constructs, GUS activity was observed as early as the closed flower bud stage, however no activity was detected in mature pollen (Fig. 2.7 B.). It seems that pollination was not required for TT10 promoter activity, because GUS activity was detected in siliques 7-days after flower castration (data not shown). On early stages of silique development (Fig. 2.7 C.) staining was present mainly in the transmitting tissue of the silique, and observed first at the top and then progressing to the bottom of the silique. Around 4-5 DAF (Fig. 2.7 D.) promoter activity was detectable in the funiculus as well as in the seed testa (endothelium layer). 2-3 days later (Fig. 2.7 E.), staining becomes very strong and the whole seeds were stained, which could be due to expression in outer integument 1 layer of the seed coat. Figure 2.7 F. is showing a silique at 8 DAF, which exhibits strong staining in aborted seeds and transmitting tissue. Stars at figure Fig. 2.7 C., E. and H. are highlighting the abscission zone of the sepals and petals. This staining is detectable shortly after petals are desiccating and sometimes is detectable as late as 14 DAF (Fig. 2.7 H.). At the later stages, GUS staining is no longer detectable in seeds which could be due to impermeability of the seed coat (Fig. 2.7 G. and H.), because in mechanically opened silique and scalpel wounded seeds, strong GUS staining is observed (Fig. 2.7 I.). In the mature siliques (Fig. 2.7 G. to I.) intensive GUS staining is visible in the dehiscence zone of the replum, but not in the valve. GUS activity is not detectable in any other tissues in plants grown in normal conditions in the greenhouse (Fig. 2.7 J. to M.). Plants grown in vitro, normally do not exhibit detectable GUS activity. Figure 2.7 N. is showing a seedling 10 days after transfer to the growth chamber, grown in the liquid 0.5x B5 medium supplemented with 0.5% sucrose. GUS activity is also not detectable in seedlings grown in vitro on vertical plates up to 14 days in long day or in constant light as well as in 10 day-old dark grown seedlings (data not shown). Other growth conditions and corresponding GUS patterns are described in section 2.4.

The pTT10_1 and pTT10_2 fragments did not exhibit detectable GUS activity in planta, except faint blue staining of the funiculus and abscission zone of the seed in pTT10_2. The 194bp (between pTT10_2 and pTT10_3) and 98bp region (between pTT10_1 and pTT10_2) are required for seed coat and funiculus promoter activity, respectively. Moreover, it seems that the region upstream from -465bp (pTT10_3) contains a sequence required for high gene expression, because much stronger GUS staining was observed for pTT10_4, pTT10_5 and pTT10_6, than pTT10_3. For those constructs, much higher concentrations of the potassium ferricyanide and potassium ferrocyanide (FF) had to be used to eliminate GUS diffusion and artefactual staining of other tissues. No FF was used and material was ethanol bleached in the organs with weak uidA activity. Similar results were obtained for the constructs studied in Ws-4 background (data not shown).
Figure 2.7: Pattern of TT10 promoter activity in wild-type plants. (A) to (H) Expression of the pTT10_3:GUS/pBIB-Hyg cassette in wild-type Col-0 plants. (A) Inflorescence of 5-week-old plant; (B) dissected flower bud squared in (A); (C) to (H) various stages of developing siliques, (C) 4 DAF, (D) 5 DAF, (E) and (F) 8 DAF: (E) normal siliques and (F) 'abnormal' siliques with aborted seeds. (G) top and (H) bottom part of a 14-DAF siliques (left) and a 18-DAF siliques (right). Arrows are pointing week GUS staining. Stars highlight GUS staining in abscission zone of the petals. (I) pTT10_5:GUS/pBIB-Hyg, siliques ±14 DAF (left) and ±18DAF (right). (J) to (N) pTT10_6:GUS/pBIB-Hyg; (J) to (M) various organs of 5-weeks-old plant, (J) stem, (K) rosette leaf, (L) root, (M) cauline leaf; (N) 10 days old seedling.

Different concentrations of potassium ferricyanide and potassium ferrocyanide were used to prevent GUS diffusion: A to H 1mM, I 2mM, J to N 0mM. Bars = 170μm B, 500μm C, 600μm D to F, 700μm G and H, 1mm J and N, 2mm A, I and K, 3mm L and M.

DAF - days after flowering
2.1. TRANSCRIPTIONAL REGULATION OF TT10 EXPRESSION

<table>
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<tr>
<th>AtTT10 promoter length</th>
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Table 2.1: Summary of TT10 promoter activity pattern in planta.
This table is summarizing promoter dissection experiment and is presenting reproducible patterns observed for each of the constructs studied in wild-type plants. Relative promoter strength is represented by number of plus (+) signs and is based on the various concentrations of potassium ferricyanide and potassium ferrocyanide used to eliminate GUS diffusion (for quantitative results see Fig. 2.8 below).

2.1.3.3 Quantitative GUS assay - MUG

To precise the different strengths of the dissected promoter fragments, quantitative measurement of the GUS activity was realized. It is possible to measure precisely GUS activity, using fluorometric assays with very small amounts of transformed plant tissue (Jefferson et al., 1987). To confirm the differences in GUS staining observed for different promoter fragments, two or three lines for each construct have been selected. Because the amount of GUS protein could depend on the number of T-DNA inserts in transgenic plants, only the lines segregating 3:1 for antibiotic resistance : sensitivity were selected (good indication for single locus of the T-DNA insertion). Considering the pattern of TT10 transcript accumulation (Fig. 2.1) and the promoter tissue specificity, 7-day-old siliques have been selected as samples to compare GUS activity.

There was a clear difference in GUS activity between the promoter fragments analysed in MUG experiment. The longest constructs pTT10_6, pTT10_5 and pTT10_4, have comparable, very high GUS activity, whereas it is 25 - 50 fold lower for pTT10_3. It is still possible to detect weak GUS activity for pTT10_2, but not for pTT10_1. Similar results were obtained for the constructs studied in Ws-4 background (data not shown).

2.1.3.4 Sub-dissection and site-directed mutagenesis - introduction

To continue, sub-dissection of the 194bp region (between pTT10_2 and pTT10_3), site-directed mutagenesis experiments were carried out to narrow down the region required for promoter activity in seeds. The ultimate goal of this part of the work was to find out which cis-regulatory elements in the promoter are functional in
vivo. The dissection experiment (2.1.3.1) defined a 194bp promoter region, which contains several putative CAREs defined in *in silico* analysis. A sub-dissection of this region was planned to further limit the promoter region and number of putative CAREs considered for site-directed mutagenesis.

![Figure 2.8: Comparison of GUS activity of 5’-dissected TT10 promoter in planta.](image)

The differences in a GUS activity, which was measured in siliques (7DAF) of T3 transformant lines in Col-0 background are shown. Promoter fragments studied and their lengths are represented by the open rectangles. Values represent averages ±SE of six technical replicates.

### Sub-dissection

Three constructs have been carried out to test putative CAREs found in the 194bp region. Looking from the 5’-end of the pTT10_3 promoter fragment, construct pTT10_3C - 412bp removes ABRE-like, DPBF and MYC2 motifs. Next fragment, pTT10_3B - 344bp, was removing GARE MYB, ACTCAT, ARR1, I-box and three DOF motifs. Finally pTT10_3A - 318bp was planned to keep the MYC2 motif and not include W-box and ABRE which are just upstream (see Fig. 2.6 for details).

### Technical difficulties encountered

The cloning strategy was planned to follow the same steps used for the promoter dissection construct preparation (2.1.3.1). Nevertheless, in this case the transfer of the promoter:GUS cassette into the *pBIB-Hyg* binary vector did not result in the appropriate constructs. To optimize cloning conditions various ratios of the insert and vector were tested. We have tried to purify the DNA fragments with several commercially available kits (see 3.2.4.2). Restriction analysis of *pBIB-Hyg* binary vector with *SmaI* and *KpnI* restriction enzymes revealed that both sites were cut with high efficiency. However the religation after single cutting was of very low efficiency with different batches of ligase. In parallel, alternative approaches have been proposed and are described below.

### Cloning in *pCAMBIA 1300*

*pCAMBIA 1300 (pCAMBIA) ([www.cambia.org](http://www.cambia.org)) is a binary vector, which is routinely used in the Seed Biology Laboratory. The analysis of the restriction sites to take out the promoter:GUS cassette from the *pBS-GUS* and those in the multi-cloning site of the *pCAMBIA*, revealed that *KpnI-PstI* are the only sites available. Briefly, the cloning protocol was as follows: i) plasmid preparation was omitting gel purification after the restriction; ii) ligation was followed by *Sall* digestion, to eliminate false positives from not completely digested binary vector; iii) after restriction and ligation the reaction mix was desalted; iv) bacterial colonies were screened for positive clones by restriction analysis (strategy based on advises from Nathalie Berger). The integrity of all the constructs had been checked by sequencing and plants were transformed.
2.1. TRANSCRIPTIONAL REGULATION OF TT10 EXPRESSION

Unexpectedly the study of the GUS pattern in T1 plants, revealed very strong blue staining in all the plant tissues tested for all the constructs. One of the hypotheses to explain such pattern of GUS activity was first association of the possible induced activity with heavy aphid/greenfly invasion in the greenhouse. However, this GUS pattern was also observed in T2, antibiotic-resistant plants after the anti-aphid treatment. Another possible explanation is the effect of the strong promoter CaMV35S, used for HptII gene expression in pCAMBIA (Fig. 2.9). This promoter, used to ensure the antibiotic resistance could also interact with the TT10 promoter fragments, which were inserted side-by-side, to drive ectopic GUS expression through its enhancers. Indeed, the pattern of GUS activity resembles the one driven by 35Sdual:GUS/pBIB-Hyg (Fig. 2.9).

Figure 2.9: GUS activity driven by the TT10 promoter in pCAMBIA binary vector.

(A) Ectopic expression of the GUS gene driven by various TT10 promoter fragments illustrated with pTT10_3:GUS/pCAMBIA1300.
(B) GUS activity driven by CaMV35S dual promoter in siliques
(C) Map of the pCAMBIA 1300 vector.
(D) Map of the T-DNA cassette showing the HptII - Hygromycin resistance gene and the CaMV35S promoter (red box). A KpnI-PstI insertion of the promoter:GUS constructs realized. LB/RB - left and right borders of the T-DNA insert. Arrow is pointing hypothesized involvement of CaMV35S enhancers in ectopic expression of the uidA gene.
CHAPTER 2. RESULTS

Cloning in pBI101GUS-GTW

Considering the unexpected results obtained with the promoter studies in the pCAMBIA vector, another alternative approach has been used to carry out the sub-dissection and site-directed mutagenesis experiments. Because of the time constraints of the thesis, the GATEWAY® cloning has been used, which allowed very fast vector preparation (Earley et al., 2006). On the other hand, a compromise had to be made and the 5'UTR sequence had to be modified to introduce the GATEWAY® recombination sites by adding 29bp at both ends of the promoter. The pBI101GUS-GTW and the cloning strategy are described elsewhere (Baudry et al., 2006; Dubos et al., 2008). Briefly, the promoter fragments have been PCR-amplified with the primers containing attB1 and attB2 GATEWAY® recombination sites (Appendix, Tab. 5.8) and introduced into the pDONR207 vector by BP recombination. The next step was the LR recombination to transfer the promoter fragment into the binary vector pBI101GUS-GTW. The integrity of all the constructs was checked by sequencing and plants were transformed. Results obtained with sub-dissection are described below in the section 2.1.3.5, together with the results of the site-directed mutagenesis.

Site-directed mutagenesis

A complementary approach to deletion analyses to check the functionality of the putative cis-acting regulatory elements found in the 194bp region (between pTT10_2 and pTT10_3), which could be required for the promoter activity in seed coat, is site-directed mutagenesis. The motifs for the analysis were chosen based on the in silico analysis described in section 2.1.2. Some of the putative CAREs are located very close from each other or even overlapping, therefore it was decided to analyze those together. The constructs have been named - M - for mutagenesis, and numbered 1 to 5 referring to the respective construct. The nucleotide substitution in the constructs prepared is based on the one which had been found in the literature describing motif identification. We checked that the mutagenised CAREs considered in the experiment, were not recognized by in silico analysis (PLACE, Signal Scan). Figure 2.6 is illustrating the location of the motifs present in the five constructs studied, with the details of the modified sequence given in the box. The mutagenesis was prepared on the full pTT10_3 promoter fragment because elements downstream -271bp (pTT10_2) could be necessary for mutual activation of GUS activity.

Construct preparation

The point mutations have been introduced in the promoter sequence using QuickChange® Site-Directed Mutagenesis Kit (Stratagene®). It is a PCR based technique which allows efficient site-specific mutation in a double-stranded plasmid (www.stratagene.com and product manual). Primers have been designed using PrimerX. Nucleotide substitutions have been confirmed by sequencing. The following, cloning steps and difficulties encountered were as described for sub-dissection construct preparation. Multiple attempts of cloning into the pBIB-Hyg did not result in the proper construct and those in pCAMBIA 1300 gave ectopic GUS activity. Finally, the constructs were prepared with the GATEWAY® technique and the preliminary results of GUS pattern in T1 plants are discussed below.

2.1.3.5 Patterns of GUS activities after TT10 promoter sub-dissection and site-directed mutagenesis

The result of the TT10 promoter sub-dissection and site-directed mutagenesis are summarized in Table 2.2. The analysis focused on seeds, where it was expected to observe blue staining for the minimal promoter, pTT10_3 - positive control, or no GUS activity in pTT10_2 - negative control. Those two constructs have been the reference of TT10 promoter activity and their comparison was required to validate the data obtained in the new vector. For the other constructs it was expected that, if the promoter fragment/motif
required for GUS expression was eliminated, the blue staining would not be observed anymore. It would be also possible that a promoter region binding negative regulator is eliminated, which would result in strong GUS staining. The same result could be observed, if a strong activation site was created by site-directed mutagenesis.

The GUS staining observed for the \( pTT10_3\text{-}GUS/pBI101\text{-}GTW \) seems to be far weaker than for the same promoter fragment in \( pBIB\text{-}Hgg \) vector. Moreover, blue staining is not detectable at the flower/early silique developmental stages, compared to Fig. 2.7, B. to D., whereas it is very weak in older siliques, compared to 2.7, E. Staining was extremely weak, even without potassium ferri cyanide and potassium ferrocyanide. No staining was detected in other plant organs. The negative control \( pTT10_2\text{-}GUS/pBI101\text{-}GTW \) gave no detectable GUS staining, whereas this promoter fragment in \( pBIB\text{-}Hgg \) was characterized by remaining, faint blue staining in the funiculus and seed abscission zone. The GUS activity seems to be abolished in all the sub-dissection constructs (3A, 3B and 3C). For the site-directed mutagenesis, faint blue staining is observed for most of the M1 and M5, T1 plants tested, whereas it seems to be detectable in less than a half of the T1 plants tested for M2. Interestingly, GUS staining was also abolished in plants carrying the M4 construct. Only 5 primary transformants have been obtained for M3 construct and GUS activity was observed in one of them.

To sum up the present results of the \( TT10 \) promoter sub-dissection and site-directed mutagenesis it has to be noted that the GUS staining for the \( pTT10_3\text{-}GUS/pBI101\text{-}GTW \) - the positive control - is much weaker than the one observed when this fragment was studied in \( pBIB\text{-}Hgg \). This could be simply due to the introduced GATEWAY® recombination site in the 5’UTR region of the promoter. If we consider the weak GUS activity of the \( pTT10_3\text{-}GUS/pBI101\text{-}GTW \) as a new reference, we could point out, that the region between \( pTT10_3 \) and \( pTT10_{3C} \) contains the motifs required for the gene expression. The lack of detectable GUS activity for most of the M2 plants could suggest that DPBF or MYB2 would be the \textit{cis}-acting elements required for \( TT10 \) gene expression. This could be further supported by the fact that the GUS activity is detectable for the constructs M1 (within 3-3C region) and M5 (downstream of 3C), which could be treated as a positive GUS staining reference. Surprisingly, no blue staining was detected for the plants carrying M4 construct what could indicate also involvement of DOF CARE. Nevertheless, all those results should be reproduced, to confirm this preliminary study.
Table 2.2: Patterns of GUS activity observed in the seed coat after sub-dissection and site-directed mutagenesis of \( TT10 \) promoter.

Table is summarizing the GUS staining observed in 5-15DAF old siliques of 12 independent T1, hygromycin resistant plants. The plus (+) sign is for the blue staining observed in seed coat. The minus (-) sign means that no GUS activity was detected in any plant organ tested (rosette leaf, cauline leaf, stem, flower and siliques). The (+) or (-) in the dashed rectangles refers to the siliques analyzed in bulk; in this case (+) stand for blue staining in most of the siliques in the well; (-) when there was no detectable GUS activity; The experiment has been repeated twice for the constructs 3, 3C, and 3B and in this case (-/+) means that GUS staining was observed in one of the repetitions. n/a - not consistent, ectopic GUS activity or plant died; for M3: plants not available. Plants were not genotyped and T2 seeds are not yet available.

2.1.4 Promoter GUS activity in protoplasts

In addition to \textit{in planta} study of the promoter activity, transient expression experiments were undertaken with protoplasts from \textit{Arabidopsis} cell suspension in order to obtain more robust quantitative analyses (see 3.2.3.8). PEG mediated transfection resulted in detectable GUS activity for all the constructs tested (1. - 5.). This approach could have accelerated the analysis, but the results obtained \textit{in planta} demonstrated that \( pTT10_1 \) and \( pTT10_2 \) do not drive detectable GUS activity. It seems that protoplasting, transfection or the culture conditions used can affect \( TT10 \) promoter activity (Takeda et al., 2002). The shortest promoter fragment, \( pTT10_1 \), contains elements sufficient for GUS expression in transfection experiment in protoplasts. Therefore it was neccesary to pursue the regulation studies \textit{in planta}.
2.2 Finding transcription factors

To progress further in the understanding of the regulation of TT10 gene expression, several approaches have been used to find candidate transcription factors which could be involved. The first strategy was to study promoter activity with reporter gene in candidate regulatory mutant backgrounds which are involved in flavonoid biosynthesis. The second approach was to select candidate transcription factors, based on co-expression data from microarrays. Several other mutants were considered on the basis of the putative cis-acting regulatory elements found in TT10 promoter, and associated with the function of the gene. Quantitative RT-PCR was used to evaluate TT10 transcript accumulation. Finally, yeast one-hybrid screen was tried to fish out some of the transcription factors binding to the promoter.

2.2.1 TT10 promoter activity in candidate regulatory mutant backgrounds

One of the ways to find out how a gene is regulated is to study promoter activity with reporter gene in a regulatory mutant background. This approach proved to be successful for BAN, which promoter activity is significantly changed in tt2, tt8 and ttg1 mutants (Debeaujon et al., 2003). The modification of the GUS pattern could be either a direct or an indirect effect. Pourcel et al. (2005) already demonstrated that the activity of the TT10 promoter is not modified in tt2, tt8 and ttg1. In this work we wanted to complete the analysis by looking whether TT10 could be regulated by the TT1 zinc finger protein, (Sagasser et al., 2002) and/or the TT16 MADS box protein, (Nesi et al., 2002) and/or the TTG2 WRKY transcription factor (Johnson et al., 2002). All those genes are involved in the regulation of flavonoid biosynthesis in seed.

To introduce TT10 promoter GUS constructs in the tt1, tt16 and ttg2 background, crosses with the plants carrying pTT10:GUS/pBIB-Hyg construct (Pourcel et al., 2005) have been made. Plants have been selected for the antibiotic resistance and seed transparent testa phenotypes, and glabra character of the leaves for ttg2. The GUS staining for pTT10:GUS/pBIB-Hyg construct in tt1, tt16 and ttg2 backgrounds, seems to be much stronger than in the control wild-type plants. Higher concentrations of the potassium ferricyanide and potassium ferrocyanide were used to prevent GUS diffusion.

Analysis of the GUS patterns revealed that in tt1 background blue staining is first detectable in the endothelium layer (Fig. 2.10 A.) and later on in the outer integument layer 1 (Fig. 2.10 D. and G.), which resembles wild-type promoter activity. The detection of GUS activity in the tt16 mutant background, at the globular stage of embryo development (Fig. 2.10 B.), is restricted to the pigment strand, but weak staining is also observed in endothelium. However, this can be due to the strong staining at the pigment strand and GUS diffusion. At the later stages, the promoter activity is visible in both endothelium and o12 seed coat layer (Fig. 2.10 E. and H.). TT10 promoter activity in ttg2 mutant background closely resembles that in tt16, with the strong staining at the micropyle and chalaza/pigment strand zones. Although the faint, patchy GUS activity was detectable in the micropyle and chalaza/pigment strand zones. Although the faint, patchy GUS activity was detectable in the endothelium (Fig. 2.10 C.). It seems that at the later stages, two-layer expression patterns is conserved (Fig. 2.10 F. and I.). The GUS activity in tt1, tt16 and ttg2 does seem not to be affected at the seed abscission zone. No ectopic promoter activity was detected in leaves nor in stem.

The results could indicate that TT10 expression might be regulated by TT1, TT16 and TTG2 in the endothelium. Nevertheless, this could be indirect regulation, because the morphology of the endothelium is affected in tt1 and tt16 mutant seeds (Nesi et al., 2002; Sagasser et al., 2002; Pourcel, 2006). In the ttg2 mutant, endothelium morphology seems not to be affected, but cells might not be finally differentiated. It has been shown that TTG2 is required for trichome development (Sagasser et al., 2002) and endosperm differentiation (Dilkes et al., 2008). Moreover, the results of the GUS staining are difficult to interpret due to the possible GUS diffusion. It would be interesting to check by in-situ hybridization, if TT10 mRNA is
detectable in the endothelium of \textit{tt1}, \textit{tt16} and \textit{ttg2} mutants.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure210.png}
\caption{Pattern of \textit{TT10} promoter activity in seeds of the candidate regulatory mutant backgrounds. Expression of the p\textit{TT10}:GUS/p\textit{BIB-Hyg} cassette in developing seeds of the candidate regulatory mutant backgrounds (\textit{tt1} - left, \textit{tt16} - middle and \textit{ttg2} - right column). Promoter activity in the wild-type is depicted by pictures adapted from Pourcel et al. (2005). (A) to (C) Seeds at the globular stage of the embryo development (3-4 DAF); (D) to (F), (H) and (I) Heart stage (6-7 DAF); (G) Bending cotyledon (8-9 DAF). GUS activity was observed with Nomarski optics on sections A to C and G to I and whole mounts D to F. az, abscission zone; C, chalaza; end, endothelium; EM, embryo; M, micropyle; oi, outer integument; PS, pigment strand. Bars = 80\,\mu m A to C; 90\,\mu m D to F, H and I; 120\,\mu m G.}
\end{figure}

2.2.2 Confirmation of \textit{TTG2} promoter activity in developing seeds

The seeds of \textit{ttg2} mutant do not accumulate PAs and mucilage, but the endothelium structure seems not to be affected. Promoter activity has been reported to be detectable in the seed coat (Johnson et al., 2002). In this study, we wanted to test the hypothesis, that \textit{TT10} expression could be regulated by \textit{TTG2}. Indeed, \textit{TTG2} encodes WRKY TF potentially binding W-box that have been found in the \textit{TT10} promoter (see 2.1.2). For that reason, we wanted to check precisely if spatio-temporal activity of both promoters are similar in developing seeds. Figure 2.11 A. is showing GUS activity driven by the \textit{TTG2} promoter, which is detectable in the endothelium and micropyle of the developing seeds at the early globular stage of embryo development. Staining seems to be very weak or absent in the chalaza - pigment strand zone. In addition,
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at the early heart stage (Fig. 2.11 B. and C.), GUS activity is detected in the mucilage cell layer. Staining at the seed abscission zone was not detected.

Activity of $TTG2$ and $TT10$ promoters seem to start at similar developmental stages and they are both detectable in the endothelium. The $TT10$ promoter activity seems to be more pronounced at the chalaza/pigment strand zone. From the heart stage of embryo development onwards, the activity of both promoters is different, with $TT10$ being in outer integument 1, whereas $TTG2$ restricted to the mucilage layer. It could be that $TTG2$ is involved in the regulation of $TT10$ in the endothelium. Another hypothesis could be that, they are both regulated by common, or distinct transcription factors resulting in the similar spatio-temporal activity. It would be interesting, to check by yeast-one hybrid and/or electrophoretic mobility shift assay if $TTG2$ could bind to the $TT10$ promoter. One more reason to verify the hypothesis of this interaction are W-boxes found in the in silico studies, which could be potential binding sites of WRKY transcription factors (see 2.1.2.2 and Fig. 2.6).

![Figure 2.11: Pattern of $TTG2$ promoter activity in wild-type seeds.](image)

Expression of the $pTTG2::GUS/pBI101.1$ cassette in developing seeds of wild-type plants. (A) Seeds at the globular stage of embryo development (3-4 DAF); (B) and (C) heart stage (6-7 DAF). GUS activity was observed with Nomarski optics on sections A and C and whole mount B. C, chalaza; end, endothelium; EM, embryo; M, micropyle; ml, mucilage layer; PS, pigment strand. Bars = 70μm A; 90μm B and C.

2.2.3 Analysis of public micro-array data - searching for co-expressed genes

The idea to look for the expression of potential regulators of the gene of interest is based on putative expression throughout time - developmental stage and space - tissue/cell. This is why we searched publicly available micro-array data to find regulatory genes co-expressed with $TT10$. Expression Angler program at BAR (http://bar.utoronto.ca/) was queried for the genes co-expressed with $TT10$ in the AtGenExpress Seed Set. First 100 top hits, regardless of the r-value were searched for the transcription factors. The list of selected co-expressed genes is presented in Table 2.3. Details of the selection are given in the legend of the table.

2.2.3.1 $TT10$ expression in candidate regulatory mutant backgrounds obtained from co-expression analysis - seed set

In the section 2.2.1 it was of interest to check if $TT10$ promoter activity was modified in previously identified regulatory mutant backgrounds. Here we decided to study the level of $TT10$ mRNA, which could indicate, if certain genes could be involved in the regulation of $TT10$ gene expression. The amount of information available for the co-expressed genes was rather scarce, therefore, we have checked, if their transcript was
present in developing flowers and/or seeds. Transcriptomic analysis (Fig. 2.12) showed that At3g15500 is strongly expressed in developing flower and seeds, but also in the senescing leaf, whereas it is below the detection level in other tissues. At2g24430 seems to be seed specific, At5g66070 is relatively ubiquitous, but high amount of transcript were detected in developing pollen, whereas At5g62320/MYB99 expression seems to be restricted to early stages of flower development. At1g71450 and At5g07700/MYB76 were below the detection level. Homozygous mutant lines have been ordered from NASC, when available. All the plants have been genotyped to confirm the presence of T-DNA insertions and an homozygous MYB99 mutant line was selected from T3 plants.

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<td>At5g66070</td>
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</table>

Table 2.3: List of selected genes co-expressed with TT10 - Seed Set.

The genes co-expressed with TT10 found with Expression Angler in the AtGenExpress Seed Set. Because of relatively low r-values, only the first five genes were listed, followed by the selection of putative transcription factors and also an other member of the laccase family. The genes with the position number in the grey background were the candidates which were studied and are described in this section.

Analysis of TT10 expression and seed flavonoid composition have been realized for the following mutants:

- At3g15500 - gene coding for ATAF-like NAC-domain transcription factor, that does not contain sequences shared by CUC1, CUC2 and NAM (Takada et al., 2001; Ooka et al., 2003).
- At1g71450 - encodes a protein containing one Apetala 2 (AP2) domain and is a member of the DREB subfamily A-4 of ERF/AP2 transcription factor family (Nakano et al., 2006). It has been shown to be involved in salt stress, cold and ABA response (Ma et al., 2006; Wenqian et al., 2007).
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- At2g24430 - gene coding for Arabidopsis NAC domain containing protein 038, Arabidopsis NAC domain containing protein 039 (Ooka et al., 2003). Induced by blue light in the cryptochrome1 mutant (Folta et al., 2003).

- At5g07700 - encodes AtMYB76 transcription factor, which has been shown to be involved in regulation of aliphatic glucosinolate biosynthesis in Arabidopsis (Stracke et al., 2001; Gigolashvili et al., 2008).

- At5g66070 - encodes a zinc finger (3HC4-type RING finger) family protein, identical to RING-H2 finger protein ATL5P (Kosarev et al., 2002), which is a ubiquitin-ligase gene significantly up-regulated after chitoctaose treatment (Libault et al., 2007).

- At5g62320 - encodes AtMYB99 transcription factor, expressed during pollen development, which has been shown to be involved in microsporogenesis (Stracke et al., 2001; Alves-Ferreira et al., 2007).

![Expression Levels of Candidate Regulators of TT10 in Developing Seeds](image)

Figure 2.12: Expression levels of candidate regulators of TT10 in developing seeds. The expression profiles were visualized with eFP Browser. Various signal (expression potential) threshold values have been set to facilitate comparison of gene expression levels. Standard deviation filtering has been used to mask samples with the deviation greater than half of their expression value (grey). Samples where expression is relatively low or considered to be below the detection level are colored in yellow. Data are not available for separate siliques (white) from torpedo stage of embryo development onwards, where only dissected seeds were analyzed. Developing seed, silique and pollen where the gene expression potential is > 100 (detectable) are painted in red.

### 2.2.3.2 Results for regulatory mutants chosen from co-expression analysis

TT10 expression in mutant backgrounds was not drastically down nor up-regulated. The largest difference was up to 25% decrease of TT10 transcript accumulation in the myb76 and myb99 mutants (Fig. 2.13).
Those results should be confirmed with a second biological replicate before further interpretation. However, the MYB transcription factor family consists of almost 130 members (Stracke et al., 2001), therefore we could expect some functional redundancy that would explain limited impact of the myb mutants (Stracke et al., 2001). The other explanation for partial decrease of the mRNA level could be the involvement of two different mechanisms of regulation in endothelium and in the outer integument 1 seed coat layer. In such a case, the absence of TT10 transcript in one layer would be masked by its expression in the other, what would be not possible to measure by RT-PCR.

Analysis of flavonoid composition (all the metabolomic analyses of flavonoid composition were done by Jean-Marc Routaboul) in the candidate mutants chosen on the basis of the co-expression data, revealed no significant differences in the amount of soluble PAs nor flavonols (Fig. 2.14). Surprisingly, in the At2g24430 mutant, we even observed a slight decrease of the soluble PAs fraction, which is in opposite to the expected tt10 mutant phenotype.

![Figure 2.13: TT10 transcript accumulation in candidate regulatory mutant background - co-expressed candidates. TT10 gene expression detected by quantitative RT-PCR, presented as a percentage of the expression of the reference gene EF1. Graph represents values for one biological repetition and averages ± SE of three technical replicates. Dark and light grey boxes represent ± 1 SE and ± 2SE values respectively. Asterixes highlight samples where TT10 expression was more than ± 1SE different from the control.](image-url)
### 2.2. FINDING TRANSCRIPTION FACTORS

#### 2.2.3.3 Co-expression analysis - developmental set

Even if *TT10* is expressed mainly in seeds, limiting the search for the co-expressed genes to the AtGenExpress Seed Set could have resulted in biased results. Indeed this data set consists in the expression data for mature seeds samples subjected to various treatments, however *TT10* is expressed during seed development. This is reinforced by the fact that in this data set, *TT10* expression potential values are very low (max. 26.83), and only few samples could be considered after standard deviation filtering. For those reasons, it would have been more appropriate to search for co-expressed genes in the AtGenExpress Developmental Set. Expression Angler program at BAR was queried for genes co-expressed with *TT10* in the AtGenExpress Developmental Set. List of selected co-expressed genes is presented in Table 2.4. It would be interesting to investigate the potential role of transcription factors which were found to be co-expressed with *TT10*. A limitation of this approach is that, if the potential candidate gene regulating *TT10* expression is not seed specific, then it will not appear in the list of the co-expressed genes, because its expression profile will not fit with the one of *TT10*. One of the top priorities would be to check the role of AtMYB5, because in the phylogenetic studies, it is in the clade together with AtMYB11, AtMYB12, AtMYB75/PAP1, AtMYB90/PAP2, AtMYB111 and AtMYB123/TT2 (Stracke et al., 2001), which have been shown to be involved in the regulation of flavonoid biosynthetic genes (Lepiniec et al., 2006; Stracke et al., 2007). AtMYB5 encodes a transcription factor that acts as a negative regulator of trichome branching and plays a role in the correct formation of the seed coat, and possibly the formation of the the underlying endosperm layers. Loss of function mutations lead to defects in seed coat mucilage, columella cells as well as trichome (Li et al., 2009) and decreased PA content (Gonzalez et al., 2008).
### Table 2.4: List of selected genes co-expressed with TT10 - developmental set.

The genes co-expressed with TT10 found with Expression Angler in the AtGenExpress Developmental Set. The first 20 genes are listed, followed by the selection of putative transcription factors position number in grey background. Other member of the laccase family have also been found (position 74).

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2.2.4 Analysis of TT10 expression in mutants involved in flavonoid biosynthesis

Although TT10 is involved in one of the last known steps of flavonoid biosynthesis pathway, it is rather considered as a modification enzyme (Pourcel et al., 2005). Nevertheless, the regulation of TT10 expression could be related to some of the known biosynthetic genes. TT10 mRNA accumulation was analyzed in the mutants known to regulate flavonol and tannin biosynthetic genes. MYB11/PFG2, MYB12/PFG1 and MYB111/PFG3 have been shown to be involved in flavonol biosynthesis in vegetative tissues (Mehrtens et al., 2005; Stracke et al., 2007). Recently MYB12 has been shown to be necessary for quercetin-3-O-rhamnoside and biflavonol accumulation, but not tannins in Arabidopsis seeds (Jean-Marc Routaboul, unpublished results). We have also included the mutant and over-expressor of AtMYB4, which encodes a transcriptional
repressor of the C4H gene, involved in the biosynthesis of flavonoid precursors (Meissner et al., 1999; Jin et al., 2000). The myb12 mutant accumulates almost 20% less TT10 mRNA than the control wild-type plants and similar results were obtained for the triple mutant myb11,12,111 (Fig. 2.15). However, the variation between the biological repetitions can reach up to 25%, therefore smaller differences should be considered as not significant before confirmation with second biological repetition. TT10 expression is almost 30% reduced in over-expressor of AtMYB4, but not modified in the myb4 mutant. This weak difference in the over-expressor, could be due to lower of the flavonoid precursor. TT10 expression in tt1, tt16 and ttg2 mutants was already analyzed (Pourcel, 2006), but semiquantitative RT-PCR did not show significant differences. We confirmed those results with quantitative RT-PCR. As explained before (section 2.2.1), TT10 expression could be regulated by those mutants in the endothelium, but we could still detect transcript present in the outer integument or the replum. The metabolomic analysis of those mutants does not correspond to the tt10 mutant phenotype, and it is described elsewhere (Routaboul et al., 2006; Stracke et al., 2007).

Figure 2.15: TT10 transcript accumulation in candidate regulatory mutant backgrounds. TT10 gene expression detected by quantitative RT-PCR is presented as a percentage of the expression of the reference gene EF1. Graph represents values for one biological repetition and averages ± SE of three technical replicates. Asterixes highlight samples where TT10 expression was more than ± 1SE different from the control.

2.2.5 Candidate genes related to stress response which could be involved in the regulation of TT10 expression

The interest in the possibility of a stress-related regulation of the TT10 emerged from the in silico analysis of the promoter sequence (section 2.1.2). Briefly, it was considered, that TT10 expression during seed development could be related to the stress response and/or protective function of the flavonoids. Expression in response to biotic and abiotic stress could depend on the crosstalk between hormone signaling pathways regulated by abscisic acid, salicylic acid (SA), jasmonic acid (JA) and ethylene, as well as ROS signaling pathways (Fujita et al., 2006). Based on this assumption and putative cis-acting regulatory elements found in the sequence of the TT10 promoter, we decided to study TT10 expression in several mutants which
have been shown to be key components of the SA, JA and ethylene signaling networks. Expression of many pathogen related (PR) genes in response to salicylic acid can be directly regulated by WRKY70 (Li et al., 2004, 2006), or mediated by NPR1. In response to pathogen attack, many other WRKY transcription factors are activated by NPR1/TGA complex (Eulgem and Somssich, 2007). We included AtWRKY23 in our study, because plants overexpressing that gene accumulate more quercetin-3-O-rhamnoside, that could indicate involvement in flavonol metabolism and link their role with stress response (Godelieve Gheysen, personal communication). Recently AtWRKY23 has been shown to be involved in pathogenesis of cyst nematode *Heterodera shachtii* (Grunewald et al., 2008). Another reason to investigate involvement of SA are recent studies, demonstrating the importance of SA for seed quality, *e.g.* protein translation, priming of seed metabolism, synthesis of antioxidant enzymes and mobilization of seed storage proteins (Rajjou et al., 2006). Part of this study was carried out with plants carrying the bacterial gene *NahG*, encoding SA hydrolase. Those plants are deprived of an active form of salicylic acid (Rajjou et al., 2006) and for that reason they were included in the present study. To question the role of the stress response which could be also mediated by jasmonic acid, two mutants were used. The first one, called *jasmonate resistant 1* (*jar1*) is impaired in JA perception (Staswick et al., 1992). Loss of function mutants are defective in a variety of responses to jasmonic acid. JAR1 is involved in pathogen defense, sensitivity to ozone, and wound responses (Lorenzo and Solano, 2005). The second one, coding for bHLH family transcription factor is AtMYC2 known as *jasmonate insensitive 1* (*jin1*). It has been shown to be a key component of the JA pathway and to act as a general integrator of different environmental stresses (Lorenzo et al., 2004; Lorenzo and Solano, 2005). We have also used the constitutive triple response1 (*ctr1*) mutant, which is a negative regulator of the ethylene signaling pathway, to check if *TT10* expression could be regulated by this hormone (Kieber et al., 1993; Guo and Ecker, 2004).

The amount of *TT10* transcript was not modified in those mutants, except for *npr1-1* and *npr1-5* where it was reduced (Fig. 2.16). Surprisingly even in the *ctr1* mutant plant, which had reduced growth phenotype and small siliques, *TT10* accumulated like in the control wild-type plants. We have reproducibly observed 50-70% decrease of *TT10* mRNA level in *npr1-1* and *npr1-5*. Surprisingly, another allele *npr1-2* is not showing any difference. However, it has to be noticed that *npr1-5* is in Nossen accession. Taking into account the natural variation demonstrated for *TT10* transcript accumulation (see 2.3.1) appropriate control for that sample is missing.

The analysis of flavonoid composition of mature seeds did not revealed any significant differences with the wild type for *wrky23*, *wrky70* and *ctr1* mutants (Fig. 2.17). Surprisingly, the *jar1* mutant accumulates less quercetin-3-O-rhamnoside and biflavonols and exhibits no significant difference in soluble PAs. It suggests that the expression of flavonol biosynthetic genes or any other process leading to their accumulation is affected in that mutant.
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Figure 2.16: TT10 transcript accumulation in candidate regulatory mutant backgrounds - stress related candidates.

TT10 gene expression detected by quantitative RT-PCR is presented as a percentage of the expression of the reference gene EF1. Values represent averages ± SE of three technical replicates. Asterixes highlight samples where TT10 expression was more than ±1SE different from the control. Experiments was repeated twice. (!) is pointing at the npr1-5 mutant which is in Nossen background.

Figure 2.17: Flavonoid composition of mature seeds of the mutants - stress related candidates.

Analysis of flavonol derivatives and tannins by LC-MS and after acid-catalyzed hydrolysis, respectively. Plants were grown in the greenhouse at the same time. Values represent averages ± SE of three independent measurements. G, glucoside; I, isorhamnetin; K, kaempferol; Q, quercetin; R, rhamnoside. The wrky23 allele is N661317.
2.2.6 Yeast one hybrid screening - fishing out transcription factors binding TT10 promoter

In parallel to the above-described approaches, a transcription factor library created by the REGIA consortium (Paz-Ares and Consortium, 2002) was planned to be screened to find transcription factors binding to the TT10 promoter. For yeast one-hybrid experiments, the pTT10_3 promoter fragment found in the dissection experiment to be required for GUS activity in the seed coat (see 2.1.3.1) was used. Briefly, the promoter fragment was PCR-amplified with primers including Smal and SacII restriction sites (Appendix Tab. 5.5) and subcloned into pCR®-Blunt II-Topo®. The Smal-SacII fragments were cloned into the MCS site of the pHISi vector in front of the HIS3 gene. Integrity of the constructs was confirmed by restriction analysis and sequencing. In the next step, Saccharomyces cerevisiae, haploid MATα yeast strain EGY48 was transformed. The procedure requires that this strain can form diploid form with the yeast strains of the REGIA library clones carrying the cDNA of the transcription factor (see 3.1.3.2 and 3.1.6). The diploid clones carrying the transcription factor binding to the sequence of interest - pTT10_3 promoter fragment, could express the HIS3 gene and grow on the selective medium. However, in many cases, one of the native yeast transcription factors can activate this expression (autoactivation). For that reason, before library screening, it is necessary to check, if yeast can grow on the selective medium. For the TT10 promoter fragment studied, all the yeast clones tested were growing on the selective medium SD-ura-his (see Fig. 2.18) Still, in the case of autoactivation, sometimes it is possible to screen the library, if the interaction is not too strong and 3-amino-1,2,4-triazole (3-AT) a competitive inhibitor of the HIS3 gene product can be used to control leaky expression of HIS3 gene. However, in the case of the TT10 minimal promoter, even 5mM concentration of 3-AT was not sufficient. To overcome this problem, smaller promoter fragments could be used. Promoter region considered to be required for the seed coat specificity and other required for the remaining GUS activity in the funiculus were cloned and tested as described above. Those two fragments also resulted in the yeast clones growing on the selective medium, even in the presence of 5mM 3-AT. The TT10 promoter fragments tested were not compatible with the yeast one-hybrid system and it was not possible to screen the REGIA library of transcription factors (Fig. 2.18).
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A. 

**promoter TT10**

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B. 

**Figure 2.18: Summary of yeast one-hybrid experiment.**

(A) Representation of the *TT10* promoter region. Selected putative cis-acting regulatory elements are presented with symbols. Alfa and Beta - promoter regions required for the seed coat and funiculus activity respectively. Primers used for cloning are represented with arrows.

(B) EGY48 yeast strain carrying *pTT10_3/pHISi* vector integrated in the *ura3* genomic region. Growth on the full (YPD) and selective medium (SD-*ura*, SD-*ura-his*). 3-AT, 3-amino-1,2,4-triazole.
2.3 Insights from natural variation

Differences for \( TT10 \) transcript accumulation among accessions have been demonstrated by Pourcel et al. (2005), showing low level of \( TT10 \) mRNA in Ler in comparison with Col-0 and Ws-4. Recently, two populations of recombinant inbred lines (RILs), Cvi-0 x Col-0 and Bay-0 x Sha were studied for flavonoid composition in mature seeds. Consistent with the results of quantitative trait locus (QTL) mapping, a gene in the \( TT10 \) interval was found to be responsible for the variation of insoluble proanthocyanidin, quercetin-3-O rhamnoside and biflavonol accumulation (Jean-Marc Routaboul, unpublished results). Three more accessions (Cvi-0, Bay-0 and Sha) were included in this study.

The objective of this part of the work, was to unravel the molecular basis of the observed differences in \( TT10 \) transcript accumulation. Gene expression and therefore promoter regions are thought to be fundamental factors in adaptive evolution. It has been demonstrated that functional changes in promoters can arise from a few mutations and that the promoter regions are major determinant of the functional genetic variation (Bentsink et al., 2006; de Meaux et al., 2006). As a starting point we assumed that differences in \( TT10 \) transcript accumulation would possibly be due to polymorphism in its promoter sequence. \( TT10 \) promoter activity was studied as well as the link between transcript accumulation and flavonoid composition.

2.3.1 Differences in \( TT10 \) transcript accumulation

The \( TT10 \) transcript accumulation was studied in 6 Arabidopsis accessions using quantitative Real Time PCR (qRT-PCR). This technique was preferred for its high sensitivity to detect small differences in gene expression. The expression of flavonoid biosynthetic genes and flavonoid accumulation are known to be affected by environmental conditions (e.g. light). For that reason, plants were grown at the same time in soil in the greenhouse in order to collect appropriate material. Open flowers were marked and siliques which were developed from them were collected 7 days after flowering (DAF). This time point was chosen because of the the first maximum of \( TT10 \) expression in siliques (see section 2.1.1 and 3.2.5).

The qRT-PCR analysis revealed that \( TT10 \) expression level is within that of the reference gene \( ELONGATION FACTOR 1alpha A4 \) in Ler, Cvi-0 and Sha accessions, whereas it was twice more in Col-0 and Ws-4 and almost twice more in Bay-0 (Fig. 2.19 A.). The differences observed cannot be explained by primer efficiency which is comparable in all accessions (Appendix, Table 3.7), nor by the differential expression of the reference gene \( EF1 \). Similar results were obtained when \( TT10 \) expression was normalized with two other reference genes, \( APT1 \) (adenine phosphoribosyltransferase) and \( TUB4a \) (tubuline beta-4 chain). Expression of the APT1 and TUB4A in siliques is presented in Appendix, Figure 3.3. The whole experiment was repeated three times resulting in nine data points used for the Student \( t \)-Test statistical analysis (Kirkman, 1996). For Ws-4, Col-0 and Bay-0, seven points were considered, because two others were out of the two standard deviation difference range from the mean. The experiment was accompanied by comparison of the developmental stages of the seeds in the silique material used for the RNA extraction (Fig. 2.19 B.). The microscopic analysis revealed no striking differences in embryo development, which were in the range of an early to late heart stage in all accessions. Plants from which samples were collected for the qRT-PCR were further grown for analysis of flavonoid composition in mature seeds (see 2.3.3).
2.3. INSIGHTS FROM NATURAL VARIATION

Figure 2.19: Assessment of $TT10$ expression levels in various Arabidopsis accessions.

(A) Expression of $TT10$ in various accessions, in siliques 7DAF measured by quantitative RT-PCR, presented as a percentage of the expression of the reference gene $EF1$. Mean and standard deviation values are given for each accession below the graph. The results of the Student $t$-Test are given for the accessions compared. Details of the analysis are in the text and section 3.2.5.

(B) Seeds were extracted from the siliques at 7 DAF and observed with Nomarski optics after mounting in chloralhydrate. The developmental stages are similar between accessions. Size bar = 90 μm.

2.3.2 Analysis of promoter sequences

To try to explain the differences observed in $TT10$ transcript accumulation, it was decided to compare the 2.0-kb region of the promoter which corresponds to the fragment studied with the GUS reporter gene. This region should contain all the cis-regulatory elements required not only for the tissue specificity, but also other elements, which could affect the level of gene expression. The primers used for the cloning of the 2.0-kb $TT10$ promoter (Pourcel et al., 2005) did not result in PCR amplification on the genomic sequence of any accession except the positive control Col-0. Multiple attempts and optimizations did not resolve this problem. For details of various modifications of the PCR conditions, different polymerases and genomic DNA extractions see 3.2.4 and 3.2.4.5.

With the set of primers used for promoter dissection (2.1.3.1), it was possible to amplify the $pTT10$ - 465bp fragment, but not the 1.0-kb or 1.5-kb. PCR amplified fragments were cloned into $pCR^{\enspace®}$-Blunt II-Topo $®$ and sequenced. Two clones from two genomic DNA extractions for each of the accessions were sequenced to result in the consensus sequences aligned in Figure 2.20 and discussed in the text below. Two other approaches used to amplify 2.0-kb or longer $TT10$ promoter region are described later (section 2.3.2.3).
2.3.2.1 Focus on the proximal part of the promoter

Analysis of the 465bp sequence of the TT10 promoter of 6 Arabidopsis accessions: Ws-4, Ler, Col-0, Cvi-0, Bay-0 and Sha, revealed no major differences. Several common single nucleotide polymorphisms (SNPs) and one in-del were found. The analysis of the motifs affected by the SNPs is based on the in silico analysis and is referring to the Col-0 sequence. Each substitution is mentioned below, with the position in the promoter, the accession and eventually the consequences it could have.

Deletion in position -384bp (Ws-4, Ler and Bay-0) is located in the MYB2 cis-acting regulatory element, nevertheless this motif is still recognized in silico in this sequence. The T to C substitution in -330bp (Ler) position results in a loss of the I-box recognition. The next SNP, G to A substitution in position -262 results in loss of the MYC2 site which was found in Col-0, but not in the other accessions. Substitution C to T at -194 could change the context of the W-box recognition in Shahdara (Sha). Two base pair variation GG changed to TT -170/169 (Ws-4, Ler, Bay-0 and Cvi-0) results in the recognition of a new element called ANAERO1CONSUSN (AAACAAAA). This motif was found in silico in promoters of 13 genes induced in anaerobic conditions (Mohanty et al., 2005). Two next SNPs common for Ws-4, Ler, Bay-0 and Cvi-0, (T to C, -158; A to T, -146) are in the region of three cis-acting regulatory elements located nearby and result in the disipation of the ACTCAT and MYB motifs, and modified context of the W-box. Substitution C to T in Ws-4 at position -100 gives a new element found in PLACE database and called LECPLEAC5 (TAAAATAT), which could be involved in ethylene response (Matarasso et al., 2005). The nearby T to A substitution -87 abolished recognition of the SORLREP3AT motif in Bay-0 accession. One SNP was found in the 5'UTR region at position +8 (G to A) and it could change the accession and eventually the consequences it could have.

One SNP was found in the 5'UTR region at position +8 (G to A) and it could change the accession and eventually the consequences it could have.

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2.3. INSIGHTS FROM NATURAL VARIATION

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CHAPTER 2. RESULTS

Figure 2.20: Promoter sequence alignment and comparison of the putative CAREs present in various accessions.
Minimal promoter (pTT10_3 - 465bp) was sequenced from six Arabidopsis accessions. Asterixes (*) are showing the single nucleotide polymorphisms (SNPs) and delta (Δ) is for the deletion. 5'UTR is underlined and transcription initiation site is marked +1. Putative cis-acting regulatory elements in the zones of the SNPs are presented, with arrows over the motif sequence, based on the Col-0 sequence analysis used as a reference. Differences due to the SNP’s are discussed in the text. (For the detailed analysis of the putative CAREs, see Fig. 2.6 and section 2.1.2).

2.3.2.2 SNP vs. TT10 transcript accumulation

It was mentioned at the beginning of this section, that functional changes in promoters can arise from a few mutations. It does not seem likely that at this point of the study, we could associate certain nucleotide variations observed among accessions with low or high level of TT10 transcript accumulation.

2.3.2.3 Analysis of long promoter fragments

The elements required for high level of gene expression could be different from the elements required for the tissue specificity of the promoter. They are located upstream the 465bp analyzed here. The results of the MUG experiment with the dissected promoter fragments (see section 2.1.3.3) support the hypothesis that the motifs required for a strong gene activation are upstream from the pTT10_3 promoter fragment. Two approaches have been used to clone 2.0-kb sequence of TT10 promoter region in various accessions.

2.3.2.4 BAC library screening

In a previous study (Pourcel et al., 2005), the TT10 promoter from Columbia accession has been amplified on the MDN11 BAC template. Here, the failure to amplify the 2.0-kb promoter fragments, could be caused by the technical difficulty of the PCR on genomic DNA. Fortunately, the Ler and Cvi libraries were available from Wim Soppe’s Group at the Plant Breeding and Genetics Department in Max Planck Institute of Cologne (MPIK). The BAC libraries were screened for the bacterial clone(s) carrying the TT10 genomic regions at the MPIK.

Briefly, two probes have been prepared, one in 5’ and one in 3’ region of the TT10 coding sequence. This approach allowed to ensure high specificity of screening, and simultaneously decreased the probability of cross hybridization with the sequence of the other member(s) of the laccase family. Screening resulted in 8 signals/bacterial clones for Cvi and 14 for Ler. Both BAC libraries have ± 10 time coverage of the Arabidopsis genome, therefore this number of hybridization signals could be an indication for good clones.

The PCR on bacterial colonies with the 3Fw/3Rev primers which are TT10 specific, gave a product of an expected size for all of them. The BAC DNA was extracted from Cvi F.22/4 and Ler D.11/3 clones. Surprisingly, the attempts to amplify TT10 promoter fragment on the BAC templates gave the same results as with the genomic, pTT10_3 being the longest fragment possible to amplify.

2.3.2.5 Going further in the promoter: amplification of longer fragments

Not being able to amplify bigger fragment than 465bp, could be due to the sequence polymorphism which was different from the one in the Col-0. Several primers have been designed upstream in the promoter region: -2.5-kb, -3.0-kb, -3.5-kb, -4.0-kb, -4.5-kb and -8.5-kb (CATMA primer in the 3'UTR of At5g48110 gene). The Col-0 genomic DNA was used as a template to validate primers and to set PCR conditions for AccuTaq(TM) LA DNA Polymerase (Sigma), which is suitable for long PCR amplifications up to 20-kb.
Finally, a single band product has been obtained for Ler with the pTT10 -4.5-kb primer and for Cvi with the At5g48110 CATMA F primer (Fig. 2.21). Both PCR products were smaller than the band obtained for the Col with the respective primer, suggesting insertion in the Col-0 sequence and/or deletions in Ler and Cvi. PCR products have been cloned into pCR®-Blunt II-Topo® for sequencing.

Sequencing of the borders of PCR fragment amplified in Ler accession revealed that the corresponding fragment contains TT10 promoter region. However, before the time when all the technical difficulties were resolved, in June 2007 comparison of the 2.2-kb Columbia and 1.5-kb Ler sequence of the TT10 promoter revealed more than 700bp deletion and several smaller in-dels differences between those accessions (Ishihara, 2007). The SNPs in the proximal 465bp were as described above.

Cloning of the PCR fragment amplified in Cvi accession was of very low efficiency, because of very big size. Sequencing of the PCR fragment in the only one clone obtained, revealed that the sequence did not correspond to the TT10 promoter region. Identity of the the fragment amplified in Cvi background should be confirmed.

**Figure 2.21: Polymorphism for length of TT10 promoter amplification.**

(A) Organization of the TT10 genomic region in Col-0 accession. 8.5-kb intergenic region is depicted with the dashed line (not in scale), the genes with open rectangles and primers with arrows indicating their orientation. For sequence of the primers, see Appendix, Tab. 5.4).

(B) Arrows (not in scale) are representing a region which is amplified and expected PCR products size is given.

(C) Agarose Gel migration of the PCR reaction and product visualization with ethidium bromide (colors inverted). n/a - no PCR amplification or smear without clear major band. PCR templates: Col - BAC MDN11; Cvi - BAC F.22/4; Ler - BAC D.11/3. Similar results were obtained on the genomic DNA of respective accessions.

### 2.3.3 Flavonoid composition in matures seeds of various Arabidopsis accessions

Previous studies uncovered many quantitative differences of flavonoid composition among accessions (Routaboul et al., 2006); Routaboul, unpublished results) which could be an adaptive trait of the plants living in different environments. Transparent Testa 10 mutant (tt10-2) has been shown to accumulate 4.6-fold more soluble proanthocyanidins (soluble PAs) than the corresponding (Ws-4) wild-type plants. The analysis of
flavonols revealed that quercetin-3-O-rhamnoside (Q-3-OR) is 50% more, whereas biflavonols are 12-fold less abundant in *tt10*-2 mature seed. Here we were trying to associate the amount of those compounds with *TT10* transcript accumulation (see section 2.3.1). Six Arabidopsis accessions, and seven *tt10* mutant alleles were grown at the same time in the greenhouse in the long day conditions. Flavonoids were extracted from freshly harvested seeds and measured, combining acid-catalyzed hydrolysis and liquid chromatography-mass spectrometry (LC-MS), as described elsewhere (Pourcel et al., 2005; Routaboul et al., 2006). The visual inspection of seed color, confirmed the pale-brown color of all the *tt10* alleles and pictures are presented in Appendix Fig. 5.2 (A.). Moreover, the Col-0 seeds seemed to be slightly darker than those of Ler and Cvi-0, whereas those of Ws-4, Bay-0 and Sha slightly brighter. The amount of all the flavonoids measured is comparable among accessions tested. The exception was Cvi-0, which accumulated much more soluble PAs, whereas almost no Q-3-OR and biflavonols. In contrast the Col-0 accession accumulated less soluble PAs and Q-3-O-R than other accessions, but the amount of biflavonols was not affected (Fig. 2.22 (A)). All the *tt10* alleles accumulates more soluble PAs and Q-3-OR, but less biflavonols, than the corresponding wild-type plants, as expected.

**Flavonoid composition vs. transcript accumulation**

Unfortunately we did not find correlation between the amount of the *TT10* mRNA (section 2.3.1 and Fig. 2.19) and flavonoid composition of the mature seeds (section 2.3.3 and Fig. 2.22).
2.3. INSIGHTS FROM NATURAL VARIATION

Figure 2.22: Flavonoid composition of mature seeds of various accessions and tt10 alleles. (A) and (B) Flavonol derivatives were analyzed by LC-MS and tannins were measured after acid-catalyzed hydrolysis. Values represents averages ± SE of three independent measurements. G, glucoside; I, isorhamnetin; K, kaempferol; Q, quercetin; R, rhamnoside.
2.3.4 Functional complementation of Ler accession with Col-0 TT10 genomic region

In this section, to assess if the level of TT10 expression in Ler could be increased by the expression of Col-0 TT10 gene, namely a 8.0-kb TT10 genomic construct which was previously used to complement the tt10 mutant (Pourcel et al., 2005). This construct contains the full TT10 coding sequence and more than 2.0-kb promoter fragment. Analysis of the independent transformant lines shows that the TT10 transcript amount can reach much higher levels than the one detected in Ler or Col-0 wild-type plants (Fig. 2.23). This could be explained by multiple T-DNA inserts. Analysis of segregation for hygromycin resistance showed that for most of the lines more than 90% of T2 plants were resistant to the antibiotic suggesting at least two loci, and only Ler C' was segregating 3:1 ratio (resistant : not resistant).

Flavonoid composition analysis revealed no decrease in the soluble PAs fraction in any of the transgenic lines (Fig. 2.24). Surprisingly, one of the lines (Ler A) contained even more soluble PAs than the control Ler wild-type plants. Four lines accumulated much less Q-3-O-R than Ler and Col-0, which could suggest that a ‘complementation’ occurs, but at the same time there was also a significant decrease in the biflavonol amount. The abundance of other flavonoids was not modified in the transformants lines. Transformant seeds at harvest seemed to be darker than those of the Col-0 wild-type plants (Appendix, Fig. 5.2).

Figure 2.23: TT10 transcript accumulation in Ler plants transformed with the Col-0 wild-type TT10 genomic region.

TT10 gene expression detected by quantitative RT-PCR, presented as a percentage of the expression of the reference gene EF1. The control wild-type plants are represented by the full bars: Ler - black, Col-0 - grey; Ler transformants - white/open bars. Values represent averages ± SE of three technical replicates. Experiment was repeated twice with the same tendency of the transcript accumulation between the lines. RNA was extracted from siliques 7DAF of the T2 antibiotic resistant plants.
2.3. INSIGHTS FROM NATURAL VARIATION

Figure 2.24: Flavonoid composition of mature seeds from Ler lines complemented with the Col-0 wild-type genomic region. The order of the lines is set from left to right according to TT10 transcript accumulation level. Analysis of flavonol derivatives by LC-MS and tannins after acid-catalyzed hydrolysis. Values represent averages ± SE of three independent measurements. G, glucoside; I, isorhamnetin; K, kaempferol; Q, quercetin; R, rhamnoside.

2.3.5 Functional analysis of the Columbia promoter in other backgrounds

To investigate further if the expression of the TT10 gene could be cis- or trans- regulated, we have studied activity of the TT10 promoter fused to the GUS protein, in other accessions. Wild-type Ler, Ws-4, Cvi-0 and Bay-0 plants were transformed with pTT10_5 and pTT10_6 promoter fragments and the GUS activity was studied revealing no qualitative differences. Moreover, the dissected promoter fragments were also studied in Ws-4 background, confirming previous quantitative results obtained in Col-0 background (see 2.1.3 and 2.1.3.1). We did not obtain any transformants for Sha.
2.4 Stress induction of the TT10 promoter activity

Several aspects, namely *in silico* analysis (see 2.1.2), strong GUS staining in aborted seed (Pourcel et al., 2005) and the putative function of TT10 (discussed in 2.1.2 and 2.2.5), motivated our interest in the hypothesized role of stress conditions regulating TT10 gene expression. Pourcel et al. (2005) and Liang et al. (2006a) discussed the differences observed for TT10 transcript accumulation in roots reported by McCaig et al. (2005), as being due to the stressing conditions during plant growth. Moreover, preliminary results with the *pTT10:GUS/pBIB-Hyg* in Ws-4, #21 plants were strongly suggesting that TT10 could be ectopically induced in certain conditions. However, many conditions tested did not result in the reproducible GUS activity with *pTT10:GUS/pBIB-Hyg* in Ws-4, #21 plants as well as other transformant lines used. Conditions of the experiments and final observations are described below.

2.4.1 Abiotic stresses

Analysis of the publicly available transcriptomic data (Genevestigator, 07.02.2007), suggested, that TT10 could be ectopically induced by various biotic and abiotic stresses. The highest upregulation of TT10 expression was found for osmotic, salt, drought, heat, wounding and oxidative stresses, whereas highest downregulation was observed in sucrose, methyl jasmonate, ABA, senescence and hydrogen peroxide treatments. However, only the logarithmic differences presenting the fold induction between the samples and controls were taken in account. Absolute values and number of repetition were not considered.

Because most of the transcriptomic data were obtained with 7 and/or 14-15 days old seedlings, that developmental stage was also used in stress experiments aiming at finding ectopic GUS activity driven by the TT10 promoter. Conditions of the experiment are described here because they are important factors which could affect ectopic GUS activity. Briefly, depending on the experiment plants were grown on either filter paper wetted with water or agar solidified B5 medium supplemented with 1% sucrose or liquid 0,5x B5 medium supplemented with 0,5% sucrose. After sowing, plates were stratified for 4 days and then grown in the phytotron at 16h light / 8h dark photoperiod, except for etiolated and constant light grown plants (Fig. 2.25). Stock solutions of hormones were prepared in ethanol or DMSO and diluted in water or culture medium. All the solutions for *in vitro* culture were filter-sterilized, except B5 medium which was autoclaved. Concentrations of hormones and other chemicals were based on the information found for the appropriate transcriptomics experiment, but also 10 to 100 times higher (Tab. 2.5).

![Figure 2.25: Stress experiments - experimental design.](image-url)

For the experiments, 8 transgenic lines were used: three carrying 2.0-kb TT10 promoter GUS construct in Col-0, four carrying 2.0-kb TT10 promoter GUS construct in Ws-4 background (including #21) and one line carrying Pro35Sdual:uidA construct as a positive control of GUS staining. Apart from treatments listed in Table 2.5, plants were also grown in constant light and darkness. For all of the conditions tested, no
replicable GUS activity was observed.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration / intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>50 - 150 - 300mM</td>
</tr>
<tr>
<td>mannitol</td>
<td>100 - 300 - 600mM</td>
</tr>
<tr>
<td>sucrose</td>
<td>100mM</td>
</tr>
<tr>
<td>(+)-catechin hydrate</td>
<td>50-500mM</td>
</tr>
<tr>
<td>(-)-epicatechin</td>
<td>50-500mM</td>
</tr>
<tr>
<td>quercetin rhamnoside hydrate</td>
<td>50-500mM</td>
</tr>
<tr>
<td>ABA</td>
<td>0.1 - 10 - 100µM</td>
</tr>
<tr>
<td>GA 4-7</td>
<td>10 - 50µM</td>
</tr>
<tr>
<td>methyl jasmonate</td>
<td>100µM - 1mM</td>
</tr>
<tr>
<td>jasmonic acid</td>
<td>100µM - 1mM</td>
</tr>
<tr>
<td>salicylic acid</td>
<td>100µM - 1mM</td>
</tr>
<tr>
<td>ethanol</td>
<td>1-5%</td>
</tr>
<tr>
<td>heat</td>
<td>30 - 40°C</td>
</tr>
</tbody>
</table>

Table 2.5: Concentration of hormones, salts and other treatments in stress experiments.

Seedlings were also submitted to progressive dehydration (Fig. 2.26), grown on the vertical plates or exposed to UV light under the flow hood and then GUS-stained within 4-24 hours after the treatment. None of those treatments resulted in clear inducible GUS pattern. However, it is possible, that TT10 promoter could be ectopically induced because the blue staining was sometimes observed. Three patterns were observed. The first one considered plants grown in multiwell plates in liquid medium (Fig. 2.27 C.), where plantlets exhibited GUS activity in the root tip. It seems that it could be nutritionally related and depend on the density of sowing and developmental stage of the plantlets. The second pattern also considered the root tip, which is very often blue-stained in the seedlings grown on filter paper (Fig. 2.26). This staining also seems to be sowing-density dependent, however it could also be related to mechanical stress and root outgrowth into the filter. The third, often observed GUS activity, concerns plantlets grown on solid medium (Fig. 2.27 D.). The blue staining was sometimes observed in plantlets grown in vitro for 14 days or more. It seems to be associated with the vitrified tissues of the seedling. Surprisingly, those GUS patterns were observed only in Col-0 background. Sometimes, faint GUS activity was also observed in the upper part of the roots of plants grown in soil (Fig. 2.27 B.). Progressive dehydration (Fig. 2.26) was established to check if the limiting water availability could result in induction of the GUS activity driven by the TT10 promoter. However, the preliminary results obtained in this system with the pTT10:GUS/pBIB-Hyg in Ws-4 (#21) did not result in any GUS induction in other transgenic lines and was later not reproducible with #21 either.

2.4.2 Wounding and biotic stresses

Many of the transgenic lines analyzed, carrying GUS construct with various lengths of the TT10 promoter sometimes result in blue staining in rosette leaves. This suggested, that TT10 could be ectopically induced by unknown factors in that organ. Rosette leaves at various stages of development were pierced with a metal needle and GUS stained within 3-48 hours after. Although, blue staining was sometimes detectable, it was never associated with the wounded tissue (Fig. 2.27 A.). Moreover, GUS activity was never detected in wounded stem nor senescing leaves (data not shown). Because all the plants were grown in the greenhouse, this exceptional spotty GUS staining could be associated with bacteria which could grow on leaves and exhibit β-glucuronidase activity.
Figure 2.26: Plantlets subjected to progressive dehydration. Ectopic expression of the \( pTT10:GUS/pBIB-Hyg \) cassette in wild-type Col-0 plants is shown. 6-day-old plantlets were subjected to progressive dehydration. Time indicates hours Petri dishes were outside the magenta box. Upper pictures demonstrate evaporation of water and drying filter paper. Bottom pictures, GUS staining of the corresponding plantlets. Arrow is pointing GUS staining in the root tip. Bar = 1mm.

Figure 2.27: Ectopic GUS activity of \( TT10 \) promoter. Ectopic expression of the \( pTT10:GUS/pBIB-Hyg \) cassette in wild-type Col-0 plants is shown. (A) Pierced wounded leaf. Circles highlight piercing in the leaf; (B) Root of 5 week-old plant grown in soil; (C) 7-day-old seedlings grown in vitro in 0.5x B5 liquid medium, 0.5% sucrose; (D) 14-days-old seedlings grown on agar solidified B5 medium. Bars = 300μm magnification in C, 2mm A and D, 3mm C, 7mm B.
2.5 Identification of laccases expressed in seeds

Several research groups are interested in the function of the laccase genes (Brown et al., 2005; McCaig et al., 2005; Cai et al., 2006; Abdel-Ghany and Pilon, 2008). However, apart from LAC15/TT10 (Pourcel et al., 2005), little is known about the their role in plant physiology. One of the secondary objectives of this thesis was to find out if other members of the laccase family, which in Arabidopsis consists of 17 members, were expressed in seeds and if they could be involved in flavonoid metabolism.

2.5.1 Expression data

The first aspect of this study was to identify the laccases which are expressed in Arabidopsis seeds. RNA was extracted from seeds of the Col-0 wild type plants dissected from siliques at 5 and 10 days after flowering (DAF). RT-PCR revealed that only LAC15/TT10 is significantly detected in Arabidopsis seeds (Fig. 2.28 A.). However in seeds at 10 DAF, a weak signal is also detected for LAC3, LAC5, LAC12, in order of decreasing signal strength. It is important to mention that a strong band of TT10 PCR product is detected with 32 cycles, whereas for other laccases even at 35 cycles only faint signal was detected (Fig. 2.28 A.).

The situation is different when RNA is extracted from the whole siliques at 10 DAF. In this case, LAC4 and LAC5 seem to be expressed at comparable levels to LAC15/TT10. A strong signal is also detected for LAC12, LAC11, LAC17, LAC3 in order of the signal strength.

We have also analyzed the expression of the laccase genes in the transcriptomic data available (Fig. 2.28 B.). The sample from the transcriptomic analysis (AtGenExpress: Developmental series), named Seed Stage 4 with/whole siliques, which corresponds to the stage of 5 DAF. Relatively strong signal in that sample, observed for LAC4 and LAC17 could come from replum and/or valve. Transcriptomic sample Seeds Stage 8 without Siliques, should correspond to the extracted seeds 10DAF (Fig. 2.28 A. and B.). Comparison of those two samples clearly confirms that LAC15/TT10 is the most expressed laccase in seeds. However, relatively strong signal was also detected for LAC5. All the other laccases seem to be not significantly expressed. It is important to note that strong signal for LAC4, LAC17 and LAC2 in the transcriptomic analysis, Seed Stage 4 with/whole siliques, is also detected by RT-PCR in siliques 3-8 DAF (data not shown). The expression remains strong for LAC4, was hardly detectable for LAC17 and below the detection level for LAC2, until 18 DAF. Later stages were not analyses (data not shown).
2.5.2 Flavonoid composition of mature seeds of the laccase mutants

*LAC15/TT10* has been shown to be involved in flavonoid metabolism and is required for the brown color of the wild-type *Arabidopsis* seeds (Pourel et al., 2005). Although it is the only laccase mutant easily distinguishable for its *tt* phenotype (Pourel et al., 2005; Cai et al., 2006), we decided to precisely analyze several laccase mutants for their flavonoid composition. Metabolomic analysis of the composition of the mature seeds confirmed the flavonol and PA phenotype of *tt10* mutant, whereas no significant differences in flavonoid composition were observed for *lac3, lac5* and *lac12* mutants (Fig. 2.29). It seems that the laccases tested are not involved in flavonoid oxidation, or their role is minor and could be masked by the presence of *TT10*. It would be interesting to analyze flavonoid composition of the laccase double mutant with *tt10*. 

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**Figure 2.28:** Comparison of the expression pattern of the laccase multigene family in seed and silique. 

(A) Expression of the laccase family genes in seeds and siliques of the wild-type Col-0 plants. cDNA diluted x100, PCR at 35 cycles, 1/5 of the reaction volume for EF was deposited. Picture was taken at the limit of signal saturation for EF, therefore the band intensities can be compared to estimate the amount of transcript. The list of primers is given in Appendix, Tab. 5.9. 

(B) Laccase multigene family expression based on publicly available transcriptomic data. Bars represent gene expression level in Seeds Stage 4 with Siliques (Black) and isolated seeds, Seeds Stage 8 without Siliques (Grey). All the bars covered by the shaded box should be considered as not significant (below detection level). Data are presented in log10 scale to highlight fold differences in the expression level of the laccase family gene members. Data used for the graph were retrieved via eFP browser and correspond to AtGenExpress: Developmental series (siliques and seeds), hybridization slides: ATGE_77_D, ATGE_77_E, ATGE_77_F, ATGE_82_A, ATGE_82_B and ATGE_82_C.
2.5. **IDENTIFICATION OF LACCASES EXPRESSED IN SEEDS**

### Flavonoids

- **Flavonol Derivatives** by LC-MS and tannins after acid-catalyzed hydrolysis. Values represent averages ± SE of three independent measurements. G, glucoside; I, isorhamnetin; K, kaempferol; Q, quercetin; R, rhamnoside.

![Flavonoid composition of mature seeds of the laccase mutants](image)

**Figure 2.29:** Flavonoid composition of mature seeds of the laccase mutants. Analysis of flavonol derivatives by LC-MS and tannins after acid-catalyzed hydrolysis. Values represent averages ± SE of three independent measurements. G, glucoside; I, isorhamnetin; K, kaempferol; Q, quercetin; R, rhamnoside.

### 2.5.3 Promoter GUS activities

Several laccase promoters were cloned in collaboration with Serge Berthet (Secondary Cell Wall and Lignification Group, INRA-IJPB, Versailles) and help from Christian Dubos. Here we present the results for those which were found to be expressed in seeds: LAC3, LAC5 and LAC12 to validate the expression data, study tissue-specific expression and investigate promoter activity, after fusion with the *uidA* reporter gene. The genomic sequences of the laccase promoters were retrieved from TAIR and used to design primers. It was considered that 2.0-kb sequence downstream from the translation initiation site of the gene should contain all the elements required for the tissue specificity of gene expression. However, in case of LAC3 and LAC12, the neighboring gene was located too close and only the sequence up to the 3’UTR of that genes were considered. The respective promoter fragments (LAC3 1.44-kb, LAC5 1.9-kb, LAC12 1.65-kb) have been PCR-amplified with the primers containing *attB1* and *attB2* GATEWAY® recombination sites (see Appendix Tab. 5.10) and introduced into the *pDONR207* vector by BP recombination. Then promoter fragments were transferred into binary vector *pBI101GUS-GTW* by LR recombination (Baudry et al., 2006; Dubos et al., 2008). The integrity of all the constructs have been checked by sequencing and plants have been transformed. Presented characterization of each of the promoter patterns is based on the analysis of the GUS activity in 8 independent transformant lines. All the plants have been genotyped for the presence of corresponding constructs. The same promoter activity pattern was observed in T2 plants. In this study we wanted to have a full overview of the GUS activities driven by the laccase promoters, therefore all the plant organs are presented. However, the focus was given on the seed GUS activity.
2.5.3.1 LAC3

The \textit{LAC3}, transcript was detected only in the seedling root (McCaig et al., 2005) and in the developing seed (this study). The 1.44-kb \textit{LAC3} promoter resulted in detectable GUS activity mainly in secondary roots (Fig. 2.30 H.) and seedling root (Fig. 2.30 I.). Localized blue staining was also detected in the sepal and petal abscission zones of the receptacle (Fig. 2.30 F. and G.). No GUS activity was detected in various stages of the flower and seed development, even when no potassium ferricyanide and potassium ferrocyanide were used.

2.5.3.2 LAC5

The \textit{LAC5} transcript seems to be abundant in the whole plant (McCaig et al., 2005). However it was hardly detectable in developing seeds and only at the late stages of silique development (see 2.5.1). The GUS activity driven by 1.9-kb \textit{LAC5} promoter seems to be associated with aging of the various plant organs (Fig. 2.31). The blue staining is not visible in young flower buds, but the progression of GUS activity is observed from the open flower stage onward, first in the sepals and later petals (Fig. 2.31 A. and B.). Strong blue staining of the stigma and filament, was observed at the open flower and young silique stage, when stamens were still attached (Fig. 2.31 B. and C.). It seems that staining intensity increases with the age of the silique (Fig. 2.31 D.), however strong GUS activity is also detected in abnormal siliques (Fig. 2.31 E. and F.) and aborted seeds (Fig. 2.31 H.). Surprisingly, some blue staining was also detectable in the funiculus of the neighboring seeds the development of which seems to be normal (Fig. 2.31 G.). Stronger staining of the funiculus was observed in old siliques (14-20 DAF). It is important to note that no GUS staining was detected in developing seeds. Faint blue signal was observed in the longitudinal sections of the stem (Fig. 2.31 L.). No GUS activity was detected in rosette and cauline leaves (Fig. 2.31 M. and N.), however in older leaves blue staining seems to colocalize with the dying/senescing tissue (Fig. 2.31 O. and P.). Strong GUS activity was observed in the hypocotyl and root of the seedling (Fig. 2.31 K.) grown \textit{in vitro}. Sometimes faint blue staining was observed in the secondary roots of the plants grown in the soil (Fig. 2.31 Q.).

2.5.3.3 LAC12

The \textit{LAC12}, transcript was detected only in the stem (McCaig et al., 2005) and at low level in developing siliques and seeds (see 2.5.1). The 1.65-kb promoter of \textit{LAC12} resulted in strong GUS activity in stem and root (Fig. 2.32 A. and J.). No GUS activity was detected in upper part of the stem and inflorescence (Fig. 2.32 B. and E.). Surprisingly, in all independent transformant lines, some of the leaves had detectable GUS activity in the main vessel (pointed by arrow at Fig. 2.32 C.) or at the border of the leaf (Fig. 2.32 D.). In seedlings blue, staining was easily visible at the hypocotyl and in patchy pattern in roots (Fig. 2.32 M.). The GUS staining in the siliques (Fig. 2.32 F. to I.) is first detectable at the abscission zone (Fig. 2.32 F., H. and magnified in I.). Some staining was also observed at the upper part of the siliques, in the replum (Fig. 2.32 G.). GUS activity was also detected at the micropyle of the seed (Fig. 2.32 L.). It seems that some GUS activity was also present in the seed coat, however it was extremely weak (Fig. 2.32 K.).

2.5.3.4 Summary

The reporter gene activity driven by the laccase promoters resembles their transcript accumulation. Even though we have detected some transcript of \textit{LAC3}, \textit{LAC5} and \textit{LAC12} in developing seeds (section 2.5.1), only promoter of \textit{LAC12} gave detectable GUS activity in seeds. It is possible, that during seed excision, the
sample was contaminated by some cells coming from replum or valve, what resulted in weak signal detected by RT-PCR.

The laccase promoter activity is compared to that observed and discussed previously for \textit{LAC15/TT10} (Tab. 2.6). It seems that \textit{TT10} is the only laccase significantly present in \textit{Arabidopsis} seeds, which would explain that a mutant in that gene results in a clear phenotype. It would be interesting to check if any other laccase driven by the \textit{TT10} promoter could oxidize flavonoids and complement \textit{transparent testa 10} phenotype. It seems that some of the function of laccases could be redundant or at least partially overlapping, however, probably due to the tissue specificity only \textit{LAC15/TT10} can oxidize flavonoids in \textit{Arabidopsis} and ensure brown color of the wild-type seeds.

<table>
<thead>
<tr>
<th>Plant Organ</th>
<th>\textit{LAC3}</th>
<th>\textit{LAC5}</th>
<th>\textit{LAC12}</th>
<th>\textit{LAC15}</th>
</tr>
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<tr>
<td>Root</td>
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Table 2.6: Comparison of laccase promoter activities.
CHAPTER 2. RESULTS

Figure 2.30: Pattern of LAC3 promoter activity in wild-type plants. Expression of the pLAC3::GUS/pBI101-GTW cassette in wild-type Col-0 plants. (A) Inflorescence of 5-weeks-old plant; (B) rosette leaf; (C) cauline leaf; (D) stem; (E) various stages of developing siliques (from left: ±6, 10, 14, 20 DAF); (F) bottom part of the siliques ±14 DAF; (G) abscission zone of the siliques, ±14 DAF; (H) root; (I) 10 days old seedling.

GUS activity was observed with binocular microscope on whole mounts except G where whole mount samples in chloralhydrite, were observed with microscope, using Nomarski optics. Potassium ferricyanide and potassium ferrocyanide were not used, except E to H, 1mM. Bars = 100μm G, 280μm F, 500μm D, 2mm A and I, 3mm B, C and E, 4mm H. DAF - days after flowering.
2.5. IDENTIFICATION OF LACCASES EXPRESSED IN SEEDS

Figure 2.31: Pattern of LAC5 promoter activity in wild-type plants. Expression of the pLAC5:GUS/pBI101-GTW cassette in wild-type Col-0 plants. (A) Inflorescence of 5 weeks old plant; (B) developing flowers and young silique, senescing sepals and petals; (C) young silique, ±2-3DAF; (D) various stages of developing siliques (from left: ±6, 10, 14, 20 DAF); (E) and (F) abnormal siliques, ±8 DAF; (G) seed ±8 DAF; (H) aborted seed; (I) top and (J) bottom part of a ±20 DAF silique; (K) 10 days old seedling; (L) stem; (M) rosette leaf; (N) cauline leaf; (O) and (P) old/senescent cauline leaf; (Q) root. Arrows are pointing weak GUS staining.

GUS activity was observed with binocular microscope on whole mounts, except, (G) and (H) where, whole mount samples in chloralhydrate, were observed with microscope, using Nomarski optics. Different concentrations of potassium ferricyanide and potassium ferrocyanide were used to prevent GUS diffusion: L and O 1mM, A to J 2mM, K, M, N, P and Q 0mM. Bars = 60μm H, 80μm G, 300μm F, 400μm I and J, 500μm E, 680μm B, 800μm L, 820μm C, 1mm O and P, 2mm A and K, 3mm D, M and N, 7mm Q. DAF - days after flowering.
Figure 2.32: Pattern of LAC12 promoter activity in wild-type plants.

Expression of the pLAC12:GUS/pBI101-GTW cassette in wild-type Col-0 plants. (A) Inflorescence of 5 weeks old plant; (B) rosette and (C) cauline leaf; (D) part of stem and cauline leaf; (F) various stages of developing siliques (from left: ±6, 10, 14, 20 DAF); (G) top and (H) bottom part of a ±10 DAF and ±14 DAF siliques respectively; (I) abscission zone of the silique, ±14 DAF; (J) root; (K) seed, ±7 DAF; (L) seed coat and (L’) dissected embryo, ±14 DAF, superposition of 2 pictures; (M) 10 days old seedling. Arrows are pointing week GUS staining.

GUS activity was observed with binocular microscope on whole mounts, except I, K, L, and L’ where, whole mount samples in chloralhydrate, were observed with microscope, using Nomarski optics. Different concentrations of potassium ferricyanide and potassium ferrocyanide were used to prevent GUS diffusion: C, D, K and L 1mM, E to J 2mM, A, B, and M 0mM. Bars = 40µm L, 50µm K, 300µm I, 500µm D, 700µm G and H, 1mm A and E, 2mm M, 3mm B and F, 7mm C and J. DAF - days after flowering.
2.6 Cell suspension as a tool to study flavonoid biosynthesis

Cell suspension maintained in the Seed Biology Laboratory was originally established from Arabidopsis Columbia accession, and is a gift from Geneviève Ephritikhine from Membrane Transport and Signalisation Group, Institut des Sciences du Végétal, CNRS, Gif-sur-Yvette. Manipulation and characterization of the cell suspension was one of the secondary objectives of this thesis. Several preliminary results are discussed in this section. The ultimate goal would be to use cell suspension as a simplified system to study flavonoid biosynthesis and metabolism.

Flavonoid pathway genes in cell suspension The first step to study flavonoid biosynthesis in the simplified system was to check which, if any, genes of the pathway are expressed in cell suspension. To prepare material for RNA extraction, cell suspension was transferred to an Eppendorf and centrifuged to remove supernatant and freeze in liquid nitrogen. However, the material prepared that way was not possible to grind due to the remaining liquid which after freezing consolidated the sample. Therefore cell lysis was further done by sonication to destroy the cells and release the nucleic acids for further extraction according to the manual of the Total RNA extraction kit (Sigma). Surprisingly, the RNA yield was comparable for sonicated and liquid nitrogen freezeed samples (Fig. 2.33 A.). It seems that freezing and normal condition lysis are sufficient to extract good quality RNA from the cell suspension used in our laboratory. RT-PCR revealed that several genes of the flavonoid biosynthetic pathway were expressed and TT10 is among them (Fig. 2.33 B.). However we did not detect chalcone synthase, which is the first enzyme of the flavonoid pathway. It would be interesting to check if any flavonoids are accumulating in the cell suspension in standard culture conditions.

To further investigate which genes of the flavonoid pathway were expressed, sequences of the primers used to prepare the probes in the CATMA arrays, were retrieved from the CATMA GST database. Those primers guarantee gene specificity and result in a 200 - 500bp PCR product. The list of the primers, for which specificity was validated on the genomic DNA is listed in Appendix, Table 5.14.

This project was discontinued, due to the focus on the analysis of the developmental and stress regulation of TT10 gene expression.
Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Plant Material

Arabidopsis thaliana L. Heynh. (thale cress) is a small flowering plant that is used as a model organism to study plant biology; it is also a model for molecular and genetic analyses of the flavonoid biosynthesis pathway (Winkel-Shirley, 2001b; Routaboul et al., 2006). Arabidopsis is a member of the Brassicaceae (mustard) family, which includes cultivated species (e.g. cabbage, cauliflower, rapeseed, radish). Small size, rapid life cycle, sequenced genome, many available protocols and other resources are making Arabidopsis a suitable tool to study gene expression. More details about Arabidopsis can be found at TAIR - http://www.arabidopsis.org/portals/education/aboutarabidopsis.jsp.

3.1.1.1 Arabidopsis thaliana - wild type accessions

On the course of evolution Arabidopsis thaliana was dispersed among many habitats, where isolated populations have accumulated mutations in their genome. The mutations which are the result of the adaptation to the environment are now used to study the molecular basis of gene expression. The wild type accessions used in our experiments are listed in the Table 3.1.

3.1.1.2 Arabidopsis thaliana - mutants

To facilitate gene discovery and to speed up progress in acquiring knowledge about plant biology, a collections of mutants has been created. In my studies, I used the collection of mutants previously studied in the laboratory, as well as new candidate mutants which were acquired at Nottingham Arabidopsis Stock Centre (NASC) and at the Versailles Arabidopsis thaliana Resource Center. Transparent testa mutant 47.1/Cvi-1 has been kindly provided by Dr. Wim Soppe MPI Cologne, Germany. After allelism test at the Seed Biology laboratory, the line was designated tt10-7. The myb4 mutant was kindly provided by Dr. Cathie Martin and all pfg mutants by Dr. Bernd Weisshaar. The laccase mutant lines lac3, lac5 and lac12 were provided by Dr. Lise Jouanin. Details of the mutant lines are listed in Tables 3.2 and 3.3.
CHAPTER 3. MATERIALS AND METHODS

### Table 3.1: Wild type accessions of *Arabidopsis thaliana* used in this work.

Several information about the wild type accessions of *Arabidopsis thaliana* are presented: i) abbreviated name of the accession, ii) place of origin and general habitat information, iii) NASC ID number. Ws-4 is the parental line for Versailles T-DNA tagged lines. Information about location and habitat for this line are assumed to be like for Ws-0 (N1602). Landsberg erecta, Ler-0 (NW20) originates from original Landsberg ecotype - La-0 (N1298) after mutagenesis. Col-0 is coming from the name of university where these *Arabidopsis* seeds were taken from, to make a stock and store accessions. It originates from the Landsberg nonirradiated population. Sha, Shahdara, called also Shakdara. m - meters

<table>
<thead>
<tr>
<th>Accession</th>
<th>Location</th>
<th>Country</th>
<th>Habitat / Altitude (m)</th>
<th>NASC ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ws-4</td>
<td>Wassilewskija / Dnjepr River</td>
<td>Belarus (USSR)</td>
<td>sandy ryefield / 100-200</td>
<td>N5390</td>
</tr>
<tr>
<td>Ler-0</td>
<td>Gorzow Wielkopolski (Landsberg)</td>
<td>Poland</td>
<td>n/a / 1-100</td>
<td>NW20</td>
</tr>
<tr>
<td>Col-0</td>
<td>Gorzow Wielkopolski (Landsberg) / Columbia</td>
<td>Poland / USA</td>
<td>greenhouse / 1-100</td>
<td>N1092</td>
</tr>
<tr>
<td>Cvi-0</td>
<td>Cape Verdi Islands</td>
<td>Cape Verde Islands</td>
<td>rocky wall with moss / 1200</td>
<td>N002</td>
</tr>
<tr>
<td>Bay-0</td>
<td>Bayreuth</td>
<td>Germany</td>
<td>fallow land / 300-400</td>
<td>N954</td>
</tr>
<tr>
<td>Sha</td>
<td>Shakdara River (Pamir)</td>
<td>Tadjikistan</td>
<td>mountains / 3400</td>
<td>N929</td>
</tr>
</tbody>
</table>

For details about the lines see: NASC at [http://arabidopsis.info/](http://arabidopsis.info/).

### Table 3.2: Alleles of the *tt10* mutant studied in this work.

Table is summarizing information about *tt10* mutant alleles. Pictures of the mutant and corresponding wild type seeds are presented in Appendix, section 5.3. SALK institute: [http://signal.salk.edu/](http://signal.salk.edu/).

<table>
<thead>
<tr>
<th>#10 mutant alleles</th>
<th>Background</th>
<th>Mutagen</th>
<th>Collection</th>
<th>NASC ID</th>
<th>Reference</th>
</tr>
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<tr>
<td>tt10-1</td>
<td>Ler-0</td>
<td>EMS</td>
<td>M.Koornneef, CS1</td>
<td>N110</td>
<td>M.Koornneef</td>
</tr>
<tr>
<td>tt10-2</td>
<td>Ws-4</td>
<td>INRA</td>
<td>Versailles, CPI13</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>tt10-3</td>
<td>Ws-4</td>
<td>INRA</td>
<td>Versailles, CQK31</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>tt10-4</td>
<td>Col-0</td>
<td>T-DNA</td>
<td>SALK_002972</td>
<td>N502972</td>
<td>Pourcel et al. 2005</td>
</tr>
<tr>
<td>tt10-5</td>
<td>Col-0</td>
<td>SALK</td>
<td>128292</td>
<td>N128292</td>
<td></td>
</tr>
<tr>
<td>tt10-6</td>
<td>Col-0</td>
<td>SALK</td>
<td>114753</td>
<td>N114753</td>
<td></td>
</tr>
<tr>
<td>tt10-7</td>
<td>Cvi-1</td>
<td>EMS</td>
<td>MPIK Cologne W.Soppe, 47.1</td>
<td>n/a</td>
<td>W.Soppe, unpublished</td>
</tr>
</tbody>
</table>

### Table 3.3: *Arabidopsis thaliana* mutant lines used in this study.

Table presents information about the mutant lines used in the study. For the clarity of the presentation, mutants presented in the table are divided in sub-groups, according to the experiments where they were used and how they are described in the Results. First column is giving the name of the mutated gene, second AGI reference number. Exact identification of the line (allele) is possible with the NASC ID. Transcription factor family, or short description of the protein is also given. The background of each mutant is indicated. Mutagen agent and when appropriate T-DNA insertion details are given. Literature refers to the first description of the mutant allele or when important features of the mutation were described. Mutant collections of the SALK and Versailles institutes were described by Alonso et al. (2003) and Bechtold et al. (1993) respectively. N.B. Mutant lines from the Versailles collection are in Ws-4 background, formerly Ws-2 as stated in publications cited.
### MATERIALS

<table>
<thead>
<tr>
<th>Mutant</th>
<th>AGI</th>
<th>Protein affected</th>
<th>Background</th>
<th>Mutagen</th>
<th>Collection / Allele</th>
<th>NASC ID</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
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<td>mutant</td>
<td>At1g34790</td>
<td>Zinc finger TF</td>
<td>Ws-4</td>
<td>T-DNA</td>
<td>Versailles -DXL6</td>
<td>n/a</td>
<td>(Debeaupjon and Lepiniec, unpublished; Sagasser et al., 2002)</td>
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<tr>
<td>tilt</td>
<td>At5g23260</td>
<td>MADS box TF</td>
<td>Ws-4</td>
<td>T-DNA</td>
<td>Versailles -DXT32; tilt6-1</td>
<td>n/a</td>
<td>(Nesi et al. 2002); Kanamycin resistant; Insertion: intron</td>
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<td>tgl2</td>
<td>At2g37260</td>
<td>WRKY TF (WRKY44)</td>
<td>Ws-4</td>
<td>T-DNA</td>
<td>Versailles -CTA18; tgl2-2</td>
<td>n/a</td>
<td>(Johnson et al., 2002, Debeaupjon et al., 2003)</td>
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<tr>
<td>pfg1/myb12</td>
<td>At2g47460</td>
<td>MYB domain TF</td>
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<td>En-1</td>
<td>Transposon</td>
<td>myb12-1f</td>
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<td>SALK_077068</td>
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<tr>
<td>myb11,12,111</td>
<td>At5g49330</td>
<td>-/-</td>
<td>Col-0</td>
<td>T-DNA</td>
<td>GABI-Kat GK291D01</td>
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<td>myb12</td>
<td>A2g47460</td>
<td>-</td>
<td>Col-0</td>
<td>En-1 / T-DNA</td>
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<td>MYB domain TF</td>
<td>Col-0</td>
<td>dSpm</td>
<td>Transposon</td>
<td>Sainsbury Lab (Meissner et al., 1999)</td>
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<td>wrky23</td>
<td>A2g47260</td>
<td>WRKY TF</td>
<td>Col-0</td>
<td>T-DNA</td>
<td>SALK_003943C</td>
<td>N661317</td>
<td>(Grunewald et al., 2008); Insertion: promoter</td>
</tr>
<tr>
<td>wrky70</td>
<td>At5g56400</td>
<td>WRKY TF</td>
<td>Col-0</td>
<td>T-DNA</td>
<td>SALK_053025</td>
<td>N553025</td>
<td></td>
</tr>
<tr>
<td>ctr1</td>
<td>At5g03730</td>
<td>RAF family of serine/threonine protein kinases</td>
<td>Col-0</td>
<td>diepoxybutane</td>
<td>ctr1-1</td>
<td>N8057</td>
<td>(Kieber et al., 1993)</td>
</tr>
<tr>
<td>jar1</td>
<td>A2g43637</td>
<td>jasmone-amido synthetase; GH3 family protein</td>
<td>Col-0</td>
<td>EMS</td>
<td>jar1-1</td>
<td>N8072</td>
<td>(Staswick et al. 1992)</td>
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<tr>
<td>myc2/jin1</td>
<td>Atlg32640</td>
<td>MYC Zinc finger TF; Jasmonate Insensitive 1</td>
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<td>T-DNA</td>
<td>SALK_017054C</td>
<td>N655309</td>
<td>(Berger et al., 1996); Insertion: exon, loss-of-function</td>
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<tr>
<td>npr1</td>
<td>At lg64280</td>
<td>NONEXPRESSER OF PR GENES 1; protein binding; also known as: sat1, rim1; similar to TF inhibitor I kappab</td>
<td>Col-0</td>
<td>EMS</td>
<td>npr1-1</td>
<td>N3726</td>
<td>(Ca o et al., 1994)</td>
</tr>
<tr>
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<td>At lg64280</td>
<td>NONEXPRESSER OF PR GENES 1; protein binding; also known as: sat1, rim1; similar to TF inhibitor I kappab</td>
<td>Col-0</td>
<td>EMS</td>
<td>npr1-2</td>
<td>N3801</td>
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<tr>
<td>npr1</td>
<td>At lg64280</td>
<td>NONEXPRESSER OF PR GENES 1; protein binding; also known as: sat1, rim1; similar to TF inhibitor I kappab</td>
<td>Col-0</td>
<td>T-DNA</td>
<td>npr1-5</td>
<td>N3724</td>
<td>(Zipfel et al., 2004); Nossen, carries a T-DNA containing the kanamycin resistance gene and the PR-1a:ms2 reporter gene</td>
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Table 3.3 Part 1.
### Table 3.3 Part 2

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<tr>
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<th>Protein affected</th>
<th>Background</th>
<th>Mutagen</th>
<th>Collection / Allele</th>
<th>NASC ID</th>
<th>Comments</th>
</tr>
</thead>
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<td>nac3</td>
<td>At3g15500</td>
<td>ATAF-like NAC-domain TF</td>
<td>Col-0</td>
<td>T-DNA</td>
<td>SALK_014331 (D)/(R)</td>
<td>N514331</td>
<td>(Takada et al., 2001); Insertion: exon</td>
</tr>
<tr>
<td></td>
<td>At1g71450</td>
<td>DREB subfamily A-4 of</td>
<td>Col-0</td>
<td>T-DNA</td>
<td>SALK_136922C</td>
<td>N667434</td>
<td>(Ma et al., 2006); Insertion: promoter</td>
</tr>
<tr>
<td>lac3</td>
<td>At3g15500</td>
<td>ATAF-like NAC-domain TF</td>
<td>Col-0</td>
<td>T-DNA</td>
<td>SALK_014331 (D)/(R)</td>
<td>N514331</td>
<td>(Takada et al., 2001); Insertion: exon</td>
</tr>
<tr>
<td>myb76</td>
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<td>MYB domain protein</td>
<td>Col-0</td>
<td>T-DNA</td>
<td>SALK_055242C</td>
<td>N662521</td>
<td>(Stracke et al., 2001); Insertion: intron</td>
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<tr>
<td></td>
<td>At5g66070</td>
<td>Zinc finger TF</td>
<td>Col-0</td>
<td>T-DNA</td>
<td>SALK_148182C</td>
<td>N658053</td>
<td>(Libault et al., 2007); Insertion: exon</td>
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<td>T-DNA</td>
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<td>N662521</td>
<td>(Stracke et al., 2001); Insertion: intron</td>
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<tr>
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<td>Laccase gene</td>
<td>Col-0</td>
<td>T-DNA</td>
<td>SALK_31901</td>
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<td>(Cai et al., 2006); Insertion: intron; loss of-function</td>
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<td>Laccase gene</td>
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<td>lac5</td>
<td>At5g07700</td>
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<td>Col-0</td>
<td>T-DNA</td>
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<td>(Cai et al., 2006); Insertion: exon; loss-of-function</td>
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<td>At2g40370</td>
<td>Laccase gene</td>
<td>Col-0</td>
<td>T-DNA</td>
<td>SALK_47455</td>
<td>n/a</td>
<td>Insertion: exon</td>
</tr>
</tbody>
</table>

**Candidate regulatory mutants from co-Laccases**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>AGI</th>
<th>Protein affected</th>
<th>Background</th>
<th>Mutagen</th>
<th>Collection / Allele</th>
<th>NASC ID</th>
<th>Comments</th>
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<td>At1g71450</td>
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<td>Col-0</td>
<td>T-DNA</td>
<td>SALK_136922C</td>
<td>N667434</td>
<td>(Ma et al., 2006); Insertion: promoter</td>
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<td>(Libault et al., 2007); Insertion: exon</td>
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<td>DREB subfamily A-4 of</td>
<td>Col-0</td>
<td>T-DNA</td>
<td>SALK_136922C</td>
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</tr>
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<td></td>
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<td>Zinc finger TF</td>
<td>Col-0</td>
<td>T-DNA</td>
<td>SALK_148182C</td>
<td>N658053</td>
<td>(Libault et al., 2007); Insertion: exon</td>
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</table>

**Candidate regulatory mutants from co-Laccases**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>AGI</th>
<th>Protein affected</th>
<th>Background</th>
<th>Mutagen</th>
<th>Collection / Allele</th>
<th>NASC ID</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>lac3</td>
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<td>Laccase gene</td>
<td>Col-0</td>
<td>T-DNA</td>
<td>SALK_31901</td>
<td>n/a</td>
<td>(Cai et al., 2006); Insertion: intron; loss of-function</td>
</tr>
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<td>lac5</td>
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<td>MYB domain protein</td>
<td>Col-0</td>
<td>T-DNA</td>
<td>SALK_63466</td>
<td>n/a</td>
<td>(Cai et al., 2006); Insertion: exon; loss-of-function</td>
</tr>
<tr>
<td>lac12</td>
<td>At5g07700</td>
<td>MYB domain protein</td>
<td>Col-0</td>
<td>T-DNA</td>
<td>SALK_47455</td>
<td>n/a</td>
<td>Insertion: exon</td>
</tr>
</tbody>
</table>
3.1. MATERIALS

3.1.1.3 Arabidopsis thaliana - transgenic plants

Transgenic lines used in this study are mentioned below.

i) Pro35Sdual:uidA used as positive control in the GUS assays (Debeaujon et al., 2003),

ii) pTTG2:GUS (line 5) to check TTG2 promoter activity in the testa during seed development (Johnson et al. (2002); provided by Dr. David R. Smyth),

iii) pTT10:GUS/pBIB-Hyg #3, #16, #21, #22; all in Ws-4 background (Pourcel et al., 2005), used as positive control for the TT10 promoter activity studies and to prepare crosses (#21, see Table 3.4D.) with the candidate regulatory mutants. pTT10:GUS/pBIB-Hyg is also referred to as pTT10_6 meaning 2.0-kb TT10 promoter.

iv) p35Sdual:MYB4 to check TT10 expression in MYB4 overexpressing plants (Jin et al. (2000); provided by Dr. Cathy Martin),

v) NahG (Ler) to analyze the involvement of SA in TT10 expression (Rajjou et al. (2006); gift of Dr. Xinnian Dong).

Transgenic lines prepared in this study are listed in the tables below:

Table 3.4: Transgenic lines generated during this work.

Tables (A) and (B) are summarizing the number of independent transformants analyzed depending on vector and accession. Each line has been genotyped for the presence of the corresponding construct and a uidA gene. T2 plant segregation for antibiotic resistance was analyzed. In bold is given the name of the construct referring to its length (see Results, section 2.1.3.1). (A) TT10 promoter dissection, (*) - (Pourcel et al., 2005), (B) TT10 promoter 'sub'-dissection and site-directed mutagenesis, (C) Ler-0 plants transformed with 8-kb genomic (Col-0) region of the TT10 gene. T2 plants were antibiotic resistant, (D) Plants with transgene pTT10:GUS/pBIB-Hyg #21 introduced by cross (see above 3.1.1.3 iii)) into tt1, tt16 and ttg2 mutants (see Table 3.3). Selection was based on antibiotic resistance and transparent testa and glabra (ttg2) phenotypes.
3.1.1.4 *Arabidopsis thaliana* - cell suspension

The cell suspension culture of *Arabidopsis thaliana* maintained at the Seed Biology Laboratory was a gift from Dr. Geneviève Ephritikhine at Membrane Transport and Signalisation Group, Institut des Sciences du Végétal, CNRS Gif-Sur-Yvette. It was originally obtained from seedlings of Columbia accession by Axelos et al. (1992). The cell suspension culture is composed by small, near-uniform clumps of cells and presents green color.

3.1.2 Bacterial Strains

3.1.2.1 *Escherichia coli*, strain DH10B

Electro-competent strain, used to multiply all the vectors and for routine cloning.

Genotype: F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λi rpsL (StrR) (Invitrogen).

3.1.2.2 *Escherichia coli*, One shot® TOP 10

Electro-competent strain, used for low-efficiency cloning and to recover vectors created by PCR in the site-directed mutagenesis experiment.

Genotype: F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara, leu)7697 galU galK rpsL (StrR) endA1 napG (Invitrogen; users manual no: 28-0126).

3.1.2.3 *Escherichia coli*, DB3.1

Electro-competent strain used to multiply GATEWAY™ vectors. Strain is resistant to CcdB.

Genotype: F- gyrA462 endA1 D(sr1-recA) mcrB mrr hsdS20(ry-, mb-) supE44 ara-14 galK2 lacY1 proA2 rpsL20(Smr) xyl-5 L- leu mtl-1 (Invitrogen).

3.1.2.4 *Agrobacterium tumefaciens*, strain 58C1pMP90

The strain has been described by Koncz et al. (1984) and is used to transform *Arabidopsis thaliana* by the floral dip technique (see 3.2.3.5). The 58C1 strain has a gene for rifampicin resistance in its genome and gentamycin resistance gene on the helper plasmid Ti (pMP90).

3.1.3 Yeast strain

3.1.3.1 *Saccharomyces cerevisiae*, strain EGY48

Strain used for yeast one hybrid screen of the REGIA bank of transcription factors (see 3.1.6).

Genotype: MATα, his3, trp1, ara3, LexAop(x6)-LEU2

3.1.3.2 Yeast strains in the REGIA transcription factor library

Detail of the yeast used to create the REGIA transcription factor library are not known (Paz-Ares and Consortium, 2002). However the clones carrying the CDSs of the transcription factors are haploid: MATα. The library screening is based on their ability to mate with the MATα (e.g. EGY48) strain to form diploid forms.
### 3.1.4 Plasmids and expression vectors

Table 3.5 presents plasmids used and created during this PhD.

<table>
<thead>
<tr>
<th>Vector name</th>
<th>Origin / provided by</th>
<th>Used for</th>
<th>Selection / resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCR®-Blunt II-Topo®</td>
<td>Invitrogen</td>
<td>blunt end PCR products cloning</td>
<td>kanamycin</td>
</tr>
<tr>
<td>pGEM-T</td>
<td>Promega</td>
<td>T-End PCR products cloning</td>
<td>B-galactosidase / ampicillin</td>
</tr>
<tr>
<td>pBS-GUS</td>
<td>Debeaujon et al., 2003</td>
<td></td>
<td>ampicillin</td>
</tr>
<tr>
<td>pTT10:GUS/pBS</td>
<td>Pourcel et al., 2005</td>
<td>template for TT10 promoter fragments amplification and site-directed mutagenesis</td>
<td>ampicillin</td>
</tr>
<tr>
<td>pTT10:GUS/pBIB-HYG</td>
<td>Becker, 1990</td>
<td>plant transformation</td>
<td>kanamycin; hygromycin in plants</td>
</tr>
<tr>
<td>pCAMBIA 1300</td>
<td><a href="http://www.cambia.org">www.cambia.org</a></td>
<td>KpnI/PstI subcloning from pBS-GUS vector and plant transformation</td>
<td>kanamycin; hygromycin in plants</td>
</tr>
<tr>
<td>pBI101GUS GTW (Baudry et al. 2006)</td>
<td>Invitrogen</td>
<td>attR1/attR2, Recombination (LR) from pDONR207 to prepare transcriptional fusions with GUS reporter gene</td>
<td>chloramphenicol / kanamycin; kanamycin in plants</td>
</tr>
<tr>
<td>pDONR207</td>
<td>Invitrogen</td>
<td>attP1/attP2 - BP Recombination - PCR fragments entry vector</td>
<td>chloramphenicol / gentamycin</td>
</tr>
<tr>
<td>pHISi</td>
<td>Clontech</td>
<td>SmalI/SacII, DNA / promoter fragments analysis in one hybrid experiments</td>
<td>Ampicilline in E.coli; Insertion in ura3 locus of the yeast, SD his selection in the EGY48 strain</td>
</tr>
<tr>
<td>pTT10_X:GUS/pTopo®</td>
<td></td>
<td>cloning of PCR fragments of TT10 promoter</td>
<td>kanamycin</td>
</tr>
<tr>
<td>pTT10_X.Y.GUS/pBS</td>
<td></td>
<td>Xhol/NcoI promoter fragments cloning in front of GUS reporter gene; transient expression in protoplasts</td>
<td>ampicillin</td>
</tr>
<tr>
<td>pTT10_X:GUS/pBIB-HYG</td>
<td></td>
<td>plant transformation</td>
<td>kanamycin; hygromycin in plants</td>
</tr>
<tr>
<td>pTT10_Y:GUS/pCAMBIA1300</td>
<td></td>
<td>plant transformation</td>
<td>kanamycin; hygromycin in plants</td>
</tr>
<tr>
<td>pTT10_Z:GUS/pDONR207</td>
<td></td>
<td>sub-cloning</td>
<td>chloramphenicol / gentamycin</td>
</tr>
<tr>
<td>pTT10_Z:GUS/pBI101GTW</td>
<td></td>
<td>plant transformation</td>
<td>chloramphenicol / kanamycin; kanamycin in plants</td>
</tr>
<tr>
<td>pTT10_3(Accession)/pTOPO®</td>
<td>this work</td>
<td>cloning of PCR fragments of TT10 promoter from accessions; sequencing</td>
<td>kanamycin</td>
</tr>
<tr>
<td>pTT10_3(Accession):GUS/pBS</td>
<td></td>
<td>Xhol/NcoI promoter fragments cloning in front of GUS reporter gene; transient expression in protoplasts</td>
<td>ampicillin</td>
</tr>
<tr>
<td>pTT10_4.5kb (Ler)/pTOPO®</td>
<td></td>
<td>cloning of PCR fragments of TT10 promoter from accessions; sequencing</td>
<td>kanamycin</td>
</tr>
<tr>
<td>pLAC3, 4, 5 and 12/pDONR207</td>
<td></td>
<td>sub-cloning</td>
<td>chloramphenicol / gentamycin</td>
</tr>
<tr>
<td>pLAC3, 4, 5 and 12/pBI101GTW</td>
<td></td>
<td>plant transformation</td>
<td>chloramphenicol / kanamycin; kanamycin in plants</td>
</tr>
</tbody>
</table>

Table 3.5: List of vectors used in this study.

Table summarizes plasmids used for molecular cloning, sequencing, transient expression and Arabidopsis transformation. Promoter - GUS constructs cloned during this work are listed as a pool, where X in the name of the construct means promoter fragments from 1 to 5 (for details see Results, section 2.1.3.1), Y is for site-directed mutagenesis constructs and control of pTT10_3, and Z = Y + negative control of pTT10_2. TT10 promoter cloned from different accessions are referring to 465bp = pTT10_3 promoter amplified on genomic DNA of Ws-4, Ler-0, Col-0, Cvi-0, Bay-0 and Sha. Promoters of the LAC3, LAC4, LACS and LAC12 were amplified on the Col-0 genomic DNA and are listed together. GATEWAY™ vectors can be multiplied only in Escherichia coli, DB3.1 strain.
Figure 3.1: Maps of the vectors used for cloning.  
(A) pBS/GUS vector used to prepare transductional fusions of the promoter and GUS reporter gene, subcloning and transient expression.  (B) Binary vector pBIB-Hyg used for transfer of the constructs in the T-DNA cassette into plants.  (C) pHISi vector used for the transcriptional fusion of the nucleotide sequence and HIS3 reporter gene.

3.1.5 Cvi and Ler BAC library at MPI, Cologne

BAC genomic library of two Arabidopsis accessions, Ler and Cvi was available in Wim Soppe lab (Max Planck Institute for Plant Breeding Research, Cologne, Germany). Briefly, libraries contain ≈10x coverage of the Arabidopsis genome. The Cvi genomic library 109 is in pIndigoBAC-5 vector (Chloramphenicol
resistance), cloned in HindIII restriction site with the average insert size 142kb. In the Ler 114 BiBAC2 (Kanamycin selection) library fragments were cloned in BamHI manner, with 162kb average size of the insert.

### 3.1.6 Transcription factor library

To identify transcription factors binding to the promoter in a yeast one hybrid experiment, a transcription factor library created by REGIA (REgulatory Gene Initiative in Arabidopsis) consortium was used. Library consists of more than 2000 clones of a normalized full length open reading frame transcription factors in a yeast (Paz-Ares and Consortium, 2002).

### 3.2 Methods

#### 3.2.1 Handling of bacteria

##### 3.2.1.1 Culture

Bacteria are grown in LB medium (Luria-Bertani: 5g·l⁻¹ yeast extract, 10g·l⁻¹ bacto-tryptone, 10g·l⁻¹ NaCl) at 37°C for *E. coli* and 28°C for *A. tumefaciens*. If needed, the medium is supplemented with antibiotics (purchased from Duchefa, Haarlem, Netherlands), as shown in Table 3.6. The medium can be liquid or solidified with 1.6 % Bacto agar.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Stock (mg/ml)</th>
<th>Solvent</th>
<th>Final concentration (mg/l)</th>
<th>Bacteria</th>
<th>Plants</th>
<th>T1 selection</th>
<th>Routine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>100</td>
<td>water</td>
<td>100</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>12,5</td>
<td>ethanol</td>
<td>12,5</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>50</td>
<td>ethanol</td>
<td>50</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Hygromycin</td>
<td>50</td>
<td>water</td>
<td>50</td>
<td>50</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kanamycin</td>
<td>100</td>
<td>water</td>
<td>100</td>
<td>100</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rifampicin</td>
<td>50</td>
<td>DMSO</td>
<td>50</td>
<td>n/a</td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.6: Antibiotics

##### 3.2.1.2 Preparation and transformation of electro-competent bacteria

A bacterial culture initiated in 5 ml LB medium is incubated overnight at 37°C, under agitation at 220 rpm. One ml of this pre-culture is used to inoculate a 500 ml LB culture which is grown until reaching the exponential phase of growth (*OD₆₀₀nm = 0.4 – 0.6*). Bacteries are centrifuged 10 min at 6000 rpm and the pellet resuspended in 500 ml 10% sterile glycerol previously cooled at 4°C. Four successive centrifugations are realized with a progressive reduction of glycerol volume used for resuspension: 250, 125, 5 and 1 ml. Bacteries are aliquoted as 10 µl fractions and stored at -80°C.

#### 3.2.2 Handling of yeast

##### 3.2.2.1 Culture

For routine culture, yeasts (*Saccharomyces cerevisiae*) are grown in YPD medium (10g·l⁻¹ yeast extract, 20g·l⁻¹ bacto-peptone; 10g·l⁻¹ glucose; Clontech, Mountain View, USA) at 28°C. The medium can be
liquid, or solidified with 2.4% Bacto agar. For transformant selection, culture is realized in minimal SD (synthetic defined) medium (Clontech) without uracil and/or histidine.

3.2.2.2 Preparation and transformation of yeast

The LIAC/SS carrier DNA/PEG method (Gietz and Woods, 2002) was applied (see The TRAFO page at http://home.cc.umanitoba.ca/~gietz/method.html for details). Briefly, 5 ml YPD culture is inoculated and grown o/n with shaking at 28°C. This culture is used to inoculate 50 ml YPD medium which is grown until reaching a cell density of \(2 \times 10^7\) cells \(\cdot\) ml\(^{-1}\) (quantity for 10 transformations). After harvest and rinsing in water, the cells are resuspended in 500 \(\mu\)l 100 mM lithium acetate (LiAc) at \(2 \times 10^9\) cells \(\cdot\) ml\(^{-1}\).

The transformation mix consists in: 240 \(\mu\)l PEG (50% w/v), 36 \(\mu\)l 1.0M LiAc, 50 \(\mu\)l SS-DNA as carrier (2.0 mg \(\cdot\) ml\(^{-1}\)), \(x\) \(\mu\)l plasmid DNA (0.1-10 \(\mu\)g), \(34-x\) \(\mu\)l sterile bidistilled water. After aliquoting in 50-\(\mu\)l fractions, cells are pelleted. 355 \(\mu\)l TRAFO mix and 5 \(\mu\)l plasmid DNA are added on the pellet and vortexed. The mix is incubated at 28°C for 30 min before being heat-shocked at 42°C for 30 min. After pelleting and resuspension in water, 2-200 \(\mu\)l of the mix is plated on selection medium.

3.2.3 Handling of Arabidopsis thaliana

3.2.3.1 Seed sterilization

The sterilization solution is prepared by dissolving a tablet containing 1.5 g active chloride (Inovchlore; Inov’Chem, Brest, France) in 50 ml distilled water and 5 ml of this solution is diluted in 45 ml 96°C ethanol (final solution exhibiting 1° active chloride). Seeds are incubated during 10 min in the sterilization solution, rinsed 2 times with 96°C ethanol and finally dried overnight under a flow hood.

3.2.3.2 Plants growth conditions in vitro

Seeds are sown on ‘Arabidopsis’ medium, containing 3·1g.l\(^{-1}\) Gamborg’s B5 medium (macro- and microelements, vitamins; Duchefa, Netherlands), 1% (w/v) sucrose, 1% (w/v) agar and 5 ml\(^{-1}\) pH indicator BCP (bromocresol purple) at 0.16%, everything being autoclaved 20 min at 120°C. Seeds are stratified during 3 days at 4°C in the dark to break dormancy and synchronize germination and afterwards transferred in a growth cabinet (16h light at 20°C / 8h dark at 18°C; 60% relative humidity; 200 \(mE\cdot m^{-2}\cdot s^{-1}\) light intensity at the Petri plate level) to get plantlets. For the analysis of antibiotic resistance, seeds are germinated on ‘Arabidopsis’ medium supplemented with relevant antibiotics, as shown in Table 3.6. Around 12 days after transfer of germination plates to light, resistant versus sensitive plantlets can be numbered.

3.2.3.3 Plants growth conditions in greenhouse

Plants are grown in soil (Tref Substrates) and regularly watered with a nutritive solution (Fertil). A photoperiod of 13h with a minimal light intensity of 100 \(mE\cdot m^{-2}\cdot s^{-1}\) is ensured by additional lightening. Temperature is around 22°C during the day and 18°C during the night. Hygrometry is not controlled.

3.2.3.4 Crosses

A flower bud from the mother plant is opened by inserting the tip of one pair of forceps between petals and sepals. All immature anthers are removed with the other pair of forceps. An anther is taken from an open, mature flower from the father plant and tapped on the stigma until covering it with pollen grains. The cross is marked with a colored thread and documented (mother, father, date, color code).
3.2. METHODS

3.2.3.5 Plant transformation by the floral dip method

Plant transformation was performed as described by Clough and Bent (1998). *Arabidopsis* adult plants were dipped into a solution containing *Agrobacterium*, 5% sucrose, and 50 ml·l⁻¹ of the surfactant Silwet (Witco, Geneva, Switzerland).

3.2.3.6 Maintenance of cell suspensions

Cells have been maintained in JPL medium (Jouanneau and Péaud-Lenoël, 1967) under continuous light at 22 °C and subcultured at one-week intervals with 10% culture in 100 ml fresh medium.

3.2.3.7 Genotyping

Genotyping of the transformant and the mutant lines was applied whenever it was necessary to screen plants for the presence of a desired insert. PCR was done with a gene-specific primer and a vector specific primer.

3.2.3.8 Transient expression of promoter:GUS constructs in *Arabidopsis* protoplasts

Protoplasts were isolated from *Arabidopsis* cell suspensions, and GUS constructs were transiently expressed in protoplasts by PEG-mediated transformation as described in Marmagne et al. (2004). Briefly, *Arabidopsis* suspension cells were digested in Gamborg’s B5 medium supplemented with 0.17 M glucose, 0.17 M mannitol, 1% cellulase, and 0.2% macerozyme. The protoplasts were purified by floatation in Gamborg’s B5 medium supplemented with 0.28 M sucrose. For transformation, 0.2 · 10⁶ protoplasts were mixed with 5 μg of plasmid DNA in a solution containing PEG 6.000 25%, 0.45 M mannitol, 0.1 M Ca(NO₃), pH 9, and incubated in the dark for 20 min. Then the PEG was washed twice with 0.275 M Ca(NO₃) and the protoplasts were re-suspended in Gamborg’s B5 medium supplemented with 0.17 glucose, 0.17 mannitol, and maintained in this medium until microscopic observation.

3.2.4 DNA methods

3.2.4.1 Isolation of plasmid DNA from *E. coli*

Plasmid DNA was purified with the Qiagen Plasmid Maxi/Midi kit (Qiagen, Hilden, Germany) or GenElute™ Plasmid Miniprep kit (Sigma, USA), following the manufacturer’s recommendations.

3.2.4.2 DNA fragments purification

DNA fragment after restriction were purified with GeneElute™ Agarose Spin Columns (Sigma), or JET-SORB (Genomed) kits according to the manual’s instructions.

3.2.4.3 Isolation of genomic DNA from plants

The protocol uses the detergent properties of CTAB (cetyltrimethylammonium bromide) leading to cell lysis and to protein and polysaccharide denaturation. Around 3 g rosette leaves are ground in liquid nitrogen and transferred in a Falcon tube containing 11 ml extraction buffer (2% CTAB w/v, 1.4M NaCl, 20 mM EDTA, 0.2% β-mercaptoethanol v/v, 100 mM Tris-HCl pH 8.0) and incubated 30 min at 60°C with shaking. Organic residues (lipids, chlorophylls, ...) are extracted with 11 ml chloroform/isoamylalcool (24:1 v/v) and eliminated by centrifugation (10 min, 10,000g). Nucleic acids from the supernatant are precipitated with 1 vol. isopropanol after o/n incubation at 4°C and centrifugation (20 min, 400g). The pellet is...
resuspended in 500 μl water and RNAs are eliminated by incubating at 37°C during 30 min with 30 μl RNaseA (Sigma) at 10 mg·ml⁻¹. After deproteinisation by centrifugation (10 min, 10.000g) in 1 vol. phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) and then 1 vol. chloroform/isoamyl alcohol (24:1 v/v), DNA is precipitated by 1 vol. isopropanol and 0.1 vol. sodium acetate, then rinsed with 70% ethanol and resuspended in 100 μl water.

3.2.4.4 Rapid DNA extraction for genotyping

This protocol was used for DNA extraction for plant genotyping. Briefly, young leaf 5-7 mm in diameter was ground in liquid nitrogen in an Eppendorf tube. 60 μl 250 mM NaOH was added and sample was boiled for one minute. To neutralize, 60 μl 250 mM HCl and 30 μl Tris-HCl 0.5 N, pH 8.0-Triton 0.25% v/v are added. Sample is boiled for one minute and centrifugated (2 min, 12000g) to pellet lysis remainings. 1 μl of the supernatant is used in standard PCR reaction.

3.2.4.5 Polymerase chain reaction (PCR)

PCR (Mullis et al., 1986) was employed to amplify DNA fragments for cloning, for genotyping of transgenic Arabidopsis plants, and for screening of transformed bacterial colonies, as well as performing site-directed mutagenesis. General cycling conditions were: 94°C to 98°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 55°C to 65°C for 30 seconds and 72°C (1kb/minute), and a final extension step of 72°C for 1 minute.

PCR for cloning

When a fragment was going to be cloned, a proof-reading DNA polymerase was used. A normal reaction of 50μl contained 1X enzyme buffer, 0.2mM of each dNTP, 10 pmol of forward primer, 10 pmol of reverse primer, 1 unit of DNA polymerase, DNA template and water. Cycling conditions for Phusion High-Fidelity DNA polymerase (Finnzymes, Finland) were: 98°C for 1 minute of initial denaturation followed by, 35 cycles of 98°C for 7 seconds, 55°C for 15 seconds and 72°C for up to 3 minutes (1kb/20 seconds), and a final extension step of 72°C for 1 minute. AccuTaq™ LA DNA polymerase (Sigma, USA) was used for amplification of long DNA fragments, with cycling conditions: 98°C for 1 minute of initial denaturation, followed by 35 cycles of 94°C for 15 seconds, 55°C for 20 seconds and 68°C for up to 20 minutes (1kb/minute), and a final extension step of 68°C for 10 minutes. Reaction buffer composition was optimized for magnesium concentration (2-4 mM) and supplemented with DMSO (1-4% final concentration) according to the manufacturer manual.

PCR on bacterial colonies

Colony PCR was applied whenever it was necessary to screen bacterial colonies for the presence of a desired insert. Colony PCR was done with a gene-specific primer and a vector specific primer. A typical 20 μl reaction consisted of 1X Taq DNA polymerase buffer, 0.2mM of each dNTP, 10 pmol of forward primer, 10 pmol of reverse primer, 0.25 unit of Taq DNA polymerase (New England Biolabs) and water. The PCR mix was distributed into reaction tubes but no template DNA was added. Instead a sterilized toothpick was used to touch a bacterial colony on a plate and then the colony was mixed with the PCR mix in each tube. Cycling conditions were almost the same as the general cycling conditions, the only change was in the first step, that was established as 95°C for 5 minutes.

3.2.4.6 PCR fragments purification

PCR fragment purification was done with GeneElute™ Agarose Spin Columns (Sigma), or MinElute PCR purification Kit (Qiagen) kits according to the manual’s instructions.
3.2. METHODS

3.2.4.7 Sequencing

Sequences were realized at the Seed Biology Laboratory, on an Applied Biosystems Abi Prism 310 Genetic Analyzer sequencer using the BigDye-terminator v3.1 chemistry.

3.2.4.8 Site-directed mutagenesis

The QuikChange® Site-Directed Mutagenesis Kit from Stratagene was used according to manufacturer instructions (from 30 ng of the original plasmid). Primers were designed using the primerX software available at the http://www.bioinformatics.org/primerx/.

3.2.5 RNA methods

3.2.5.1 RNA isolation and cDNA synthesis

Frozen tissues were ground in liquid nitrogen and total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer’s instructions, completed with a on-column DNase treatment (Qiagen DNase ’Rnase-free’) to avoid DNA contamination. The only change to the protocol was addition of polyvinylpyrrolidone (Sigma) for tissue grounding. The RNA extracts were treated with 30 U of RNase-free DNase I (Qiagen) and were eluted with 40 μl of diethyl pyrocarbonate-treated water. For RT-PCR studies, DNA-free RNA was converted into first-strand cDNA using the SuperScript pre-amplification system for first-strand cDNA synthesis (Gibco BRL) with oligo(dT)22.

N.B. For the purpose of reliable results, high quality RNA is obligatory. RNA integrity was checked on agarose gel and ratio of the 28S/18S has been verified, and only samples with ratio above 1.8:1 were used. Quantification of the RNA was made with NanoDrop (Thermo scientific, Fisher).

To ensure reliable comparison of gene expression between Arabidopsis accessions and mutants open flowers were marked and siliques at the same developmental stage were collected (Fig. 3.2). For all comparison studies plants were grown in the greenhouse at the same time. Samples, were collected from three plants, except At5g62320/MYB99 where only one homozygous plant was available. RNA was extracted from three siliques from three different plants, collected and immediately frozen in liquid nitrogen. In each experiment the same amount of RNA was used for cDNA synthesis. Three technical replicates of qRT-PCR were realized for each cDNA synthesis.

3.2.5.2 RT-PCR

The cDNA samples were diluted 10- and 100-fold, and 1 μl of dilution was amplified in a 20-μl standard PCR mixture. First expression of the control gene EF1 was checked and that depending on the gene expression level, PCR reactions were carried out on different cDNA dilutions.

3.2.5.3 Quantitative real-time RT-PCR (qRT-PCR)

qRT-PCR was carried out as described in Baud et al. (2004), the reaction was performed on the LightCycler Instrument (Roche, Meylan, France) with the LightCycler-FastStar DNA Master SYBR Green I kit for PCR (Roche) according to the manufacturer’s protocol. Each reaction was performed with 5 μl of 1:10 to 1:100 dilution of the first cDNA strands in a total volume of 20 ml. The reactions were incubated at 95 °C for 8 min to activate the hot start recombinant Taq DNA polymerase, followed by 45 cycles of 10 s at 95 °C, 6 s at 55 °C, and 20 s at 72 °C. The primer set for the TT10 gene, namely 3Fw-TT10 and 3Rev-TT10 (see Appendix, Tab. 5.11) was previously designed by Lucille Pourcel (unpublished results). The specificity of
the PCR amplification was checked with a heat dissociation protocol (from 65 °C to 95 °C) following the final cycle of the PCR. PCR products were then purified and sequenced (Pourcel, unpublished). The efficiency of the primer set was calculated by performing real time PCR on several dilutions of first strands (see section 3.2.5.5). The results obtained for the different tissues analyzed were standardized to the constitutive EF1 gene expression level (Liboz et al., 1990). We confirmed that the level of EF1 expression was comparable between accessions studied by analysis of expression of two other reference genes (see 3.2.5.4).

![Figure 3.2: Flower marking and silique sample collection.](image)

Sample preparation for TT10 expression comparison between accessions. Arabidopsis accessions used in the study were developing different number of siliques per day. Picture is showing different height of stem and siliques developed in 23 days counted from first flower appearance. Arrows are showing siliques 7 days after flowering. Bar = 2cm

### 3.2.5.4 Normalization controls for qRT-PCR

In qRT-PCR, expression of the gene of interest is presented as an amount of the control gene, which amount is thought to be equal among the samples (e.g. tissues; accessions in this study). Here we demonstrate, that the expression of two other reference genes is comparable between the samples. We can assume that together with Elongation Factor 1α4, (At1g07920, At5g60390) they are reliable control genes and can be used for normalization of gene expression in qRT-PCR experiment.

### 3.2.5.5 Primer efficiency

Quantitative RT-PCR results could be artefactual due to the polymorphism in the primer binding sites and/or secondary structures of the cDNA. By showing that the primer efficiency is comparable between the samples, we assumed that the amplification was not affected by those factors in Arabidopsis accessions analyzed.
3.2. METHODS

The expression of two reference genes, APT1 (adenine phosphoribosyltransferase, At1g27450) and TUBULINE (tubuline beta-4 chain, At5g44340) have been expressed as a percentage of the Elongation Factor 1αA4 gene expression.

Table 3.7: Efficiency of the 3Fw/3Rw set of primers.

The efficiency of the 3Fw/3Rw set of primers is comparable and close to 100% in all the Arabidopsis accessions tested. The efficiency was calculated by the curve fitting for the pair of primers used to study TT10 gene expression. The highest cDNA dilution sample (x100000) was not included.
3.2.6 Histochemical methods for GUS detection and quantification

3.2.6.1 Histochemical detection of GUS activity

The histochemical analysis of GUS activity was performed as described by Debeaujon et al. (2003). Tissues were prefixed in 90% (v/v) ice-cold acetone:water for 30 min at 4°C. After three rinses in 0.1 M phosphate buffer, pH 7.2 (PB), they were transferred in a PB solution containing 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Duchefa, Haarlem, The Netherlands), 0.1% (v/v) Triton X-100:water, 0.5 to 10 mM each of potassium ferrocyanide and potassium ferricyanide, depending on the construct, and 10 mM Na-EDTA. Vacuum was applied for 1 h before incubating for 12 h at 37°C in the dark. Chlorophyll was removed by room temperature incubation in 70% (v/v) ethanol:water. Afterward, stained tissues were either cleared with a chloral hydrate solution (chloral hydrate:distilled water:glycerol (8:2:1,w/v/v) as whole mounts or embedded in resin after fixation. Namely, developing seeds for resin embedding were fixed in 2% (v/v) glutaraldehyde and 1% (v/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. After incubation at 4°C for 24 h, samples were washed in phosphate buffer, dehydrated using a series of graded ethanol solutions, and embedded in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer’s instructions. Sections (4-7 μm) were made on a rotary Jung RM 2055 microtome (Leica Microsystems, Heidelberg, Germany) equipped with metallic blades (Heraeus Kulzer). Observations were performed with an Axioplan 2 microscope (Zeiss, Jena, Germany) equipped with bright-field optics. Microscopic observations were performed with Nomarski differential interference contrast optics.

3.2.6.2 Quantification of GUS activity (MUG assay)

The fluorometric assay was performed as described by Jefferson et al. (1987) on extracts made from transgenic and control plants. The reaction product of the assay, namely 4-methylumbelliferone, was measured kinetically, in duplicate, using a fluorometer (Fluoroskan II; Labsystems, Helsinki, Finland). Protein concentration was estimated for each extract using the method of Bradford (1976).

Results of measured GUS activity are expressed in pmol/min/mg protein and are representation of experiment which was repeated twice with the same tendency. For each construct 2 or 3 independent transformant lines were studied. Two samples for each line were analyzed separately (sample consist of three siliques of the same age collected from 3 plants). Standard deviation bars are representation of the differences for all the samples of the certain construct.

3.2.7 Analysis of seed flavonoids

The results of metabolomic analysis of flavonoid composition presented, was done by Jean-Marc Routaboul. The analysis of flavonoid composition from mature seeds was modified from Routaboul et al. (2006) as follows. Fifteen mg of seeds were ground for 1 min at maximum speed using a FastPrep-24 sample preparation system (MP Biomedical) in 2ml-tube containing 1 1/4 inch ceramic sphere and 1 ml acetonitrile / water (75:25; v/v). For some experiments, a solvent designed to maximize procyanidins extraction was used (MeOH / Acetone / water / TFA, 32:40:28:0.05; v/v/v/v). Samples were sonicated for 20 min at 4°C and centrifuged at 13000rpm for 10 min. Following centrifugation, the pellet was extracted further with 1 ml acetonitrile / water (75:25; v/v) overnight at 4°C. The two extracts were pooled. An aliquote of the final extract was filtered on a 0.45μM syringe filter before being analyzed by mass spectrometry. The pellet was preserved for insoluble PA analysis.

Analyses of soluble and insoluble PAs were carried out according to Porter et al. (1986), with minor changes, on 500 μl aliquots of the final extracts and on the entire remaining pellets, respectively. Three ml
of butanol–HCl reagent (butanol / concentrated HCl, 95:5, v/v) and 0.1 ml of the ferric reagent (2% ferric ammonium sulfate in 2N HCl) were added. Soluble anthocyanins were then measured at 550 nm. The tubes were put in a boiling water bath adjusted at 98°C for 60 min. After cooling the tube on ice, PA-related absorbance was recorded at 550 nm. The amount of PAs or anthocyanins are expressed in mg·g\(^{-1}\) cyanidin equivalent using a calibration curve.

LC-MS analysis was conducted using a 'Quattro LC' with an ESI 'Z-spray' interface (MicroMass Co, Manchester, UK), MassLynx software, an Alliance 2695 RP-HPLC system (Waters, USA), and a Waters 2487 UV detector set at 280 nm. An Uptisphere C18 column (150x2 mm, 5 μm, Interchrom) was used with a mix comprising solvent A acetonitrile / water, (95:5, v/v, 0.2% acetic acid) and solvent B acetonitrile / water (5:95, v/v, 0.2% acetic acid) with a gradient profile (starting with 10:90, A/B, v/v, for 5 min; linear gradient up to 70:30, A/B over 35 min; a washing step 100:0, A/B for 15 min and final equilibration at 10:90, A/B for 20 min) at a flow rate of 0.2 ml/min. Relative quantification was based on the area of major MS signals. Flavonol contents were expressed relative to quercetin-3-O-rhamnoside and rutin ( Extrasynthese, France) for mono glycosylated and di-glycosylated flavonols, respectively. ESI source parameters were optimized using these standards.

3.2.8 Routine techniques

Routine techniques, such as DNA agarose gel, DNA precipitation, DNA ligation, DNA cleavage with restriction endonucleases and DNA concentration measurement were done according to Sambrook and Russel (2001).

3.2.9 Internet resources and services

3.2.9.1 The Arabidopsis Information Resource - TAIR
http://www.arabidopsis.org/

TT10 at TAIR

3.2.9.2 The Arabidopsis Thaliana Integrated Database - ATIDB
http://www.atidb.org/

TT10 at ATIDB

3.2.9.3 Integrative database around plant genomes - FLAGdb++ v.3.9
http://urgv.evry.inra.fr/FLAGdb (Samson et al., 2004)

3.2.9.4 The Complete Arabidopsis Transcriptome Micro Array database - CATMA Database
http://www.catma.org/

CATMA Database (Crowe et al., 2003)

3.2.9.5 AtcisDB at AGRIS
http://arabidopsis.med.ohio-state.edu/AtcisDB/

AtcisDB is the Arabidopsis cis-regulatory element database at Arabidopsis Gene Regulatory Information Server. It can be searched for cis-acting regulatory elements with AtcisDB Search Engine (Molina and Grotewold, 2005; Palaniswamy et al., 2006).
3.2.9.6 The Genevestigator - expression database and meta-analysis system

https://www.genevestigator.com/gv/index.jsp

The database has been first queried in January 2006, then systematically verified. (Last updated: 7.04.2009). (Zimmermann et al., 2005; Hruz et al., 2008).

3.2.9.7 The Bio-Array Resource for Arabidopsis Functional Genomics - BAR

http://bar.utoronto.ca/ (Toufighi et al., 2005)

Arabidopsis eFP Browser

http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi  eFP Browser is a visualization tool which pictures gene expression for easier interpretation. More over, presented data are normalized, signal threshold can be set up and standard deviation filtering can be used to keep only statistically significant results. Signal values lower than 100 (expression potential) should be considered as background (Bassel, personal communication; Dubos, personal communication). (Schmid et al., 2005; Kilian et al., 2007; Winter et al., 2007).

Expression Angler

http://bar.utoronto.ca/nfloats/cgi-bin/nfloats_expression_angler.cgi  Expression angler is program calculating the correlation coefficients for expression for all gene expression vectors compared to the gene of interest. (Toufighi et al., 2005).

3.2.9.8 GeneCAT - Gene Co-expression Analysis Toolbox

http://genecat.mpg.de/cgi-bin/kinitiator.py

GeneCAT is composed of 5 tools (e.g ExpressionProfiling, ExpressionTree, Co-expression analysis) for gene expression analysis (Cite Mutwil)

3.2.9.9 DIURNAL

http://diurnal.cgrb.oregonstate.edu/

The DIURNAL search tool provides easy access and visualization of circadian/diurnal gene expression data. (Mockler et al., 2007).

Data from ATH1 chip were normalized with gcRMA method (Wu et al., 2004).

3.2.9.10 PLACE and Signal Scan - A Database of Plant Cis-acting Regulatory DNA Elements

http://www.dna.affrc.go.jp/PLACE/signalscan.html

SIGNAL Scan (Prestridge, 1991)is a computer program that scans DNA sequences for known transcriptional elements based on the PLACE database (Higo et al., 1999). Last update of PLACE database: 08.01.2007.

3.2.9.11 Plant CARE


Plant Cis-Acting Regulatory Element database and a portal to tools for in silico analysis of promoter sequences (Lescot et al., 2002).
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3.2.9.12 Plant PAN - Plant Promoter Analysis Navigator

http://plantpan.mbc.nctu.edu.tw/gene_group/index.php

Plant PAN is a plant promoter analysis navigator, for identifying combinatorial cis-regulatory elements (Chang et al., 2008).

3.2.9.13 ATHENA

http://www.bioinformatics2.wsu.edu/Athena

Athena is a web-based application with large set of data visualization, mining, and analysis tools related to the control of gene expression (O’Connor et al., 2005).

3.2.9.14 Basic Local Alignment Search Tool - BLAST


3.2.9.15 Multiple Sequence Alignment

http://bioinfo.genotoul.fr/multalin/multalin.html (Corpet, 1988)

3.2.9.16 PrimerX - Primer design for the site directed mutagenesis

PrimerX is a program for automated design of mutagenic primers for site-directed mutagenesis. http://www.bioinformatics.org/primerx/
Chapter 4

Discussion, Conclusions and Perspectives

4.1 Regulation of \( TT10 \) gene expression

The modalities of gene expression and regulation of gene expression are related to the gene function. Housekeeping genes are typically constitutively expressed at relatively constant level across many or all known conditions. On the other hand, inducible genes are the ones, which expression is either responsive to the cell cycle, developmental stage or environmental change. Those regulation systems would ensure, that the functional protein is produced where and when it is needed. In case of \( TRANSPARENT \ TESTA \ 10 \) gene, it seems that the expression is tightly regulated during plant development and is mainly restricted to developing seeds and siliques. It has been shown that \( TT10 \) is expressed in two layers of the seed coat, where it colocalizes with, and is involved in metabolism of flavonols and tannins (Pourcel et al., 2005; Pourcel, 2006). It has also been reported that the \( tt10 \) mutant exhibits a delay in root elongation and seeds accumulated nearly 30% less extractable lignin than the wild type (Liang et al., 2006a). In the present study we were mainly interested in how \( TT10 \) expression is regulated during plant development, however previously raised concept of stress induced regulation (Pourcel et al., 2005; Pourcel, 2006; Liang et al., 2006a) was also investigated.

4.1.1 Transcript accumulation

Previous studies reported \( TT10 \) transcript in root, leaf, stem and flowers (McCaig et al., 2005; Cai et al., 2006; Liang et al., 2006a; Abdel-Ghany and Pilon, 2008), however in the present study we were able to detect \( TT10 \) mRNA only in flower, developing siliques and seeds, what is consistent with the results of Pourcel et al. (2005). Surprisingly, we have found that during silique development expression was bi-phasic with the maximum of transcript accumulation at 7 and 15 DAF. This feature could be explained by gene expression, first in the endothelium layer, and second peak could be due to the expression in the outer integument 1 (oil). However, promoter activity studies revealed, that GUS staining in oil was already detectable at 8 DAF (Pourcel et al. (2005), and this study). Most probably, the first peak of expression could reflect \( TT10 \) expression in seeds, whereas the second peak could come from the expression in the valve or replum, because RNA was extracted from the whole siliques (without receptacle and peduncle). Another possible explanation for the intermediate decrease of transcript amount, would be the massive transcriptional reprogramming of gene expression, which is observed during the transition stage of the seed development (Borisjuk et al., 2004). Moreover, Ruuska et al. (2002) have shown several distinct expression patterns of
gene expression during *Arabidopsis* seed filling. It would be interesting to compare *TT10* expression profiles in dissected seeds and other components of the siliques to find out if the observed bi-phasic expression is due to the different time of expression in those organs.

### 4.1.2 Developmental vs. stress regulated expression

Quantitative RT-PCR used in this study did not reveal any *TT10* transcript accumulation in other tissue than siliques and seeds. Previously observed expression in other organs (Pourcel et al., 2005; Liang et al., 2006a) may be due to some 'stress' or different plant culture conditions. McCaig et al. (2005) could detect *TT10* mRNA in leaves and seedling, using 40 cycle PCR amplification. However, this system set for high sensitivity of transcript detection, could result in artifacts. In comparison, the 35-cycle PCR used here to study the expression profiles of the laccase family genes in seeds, revealed that *TT10* is the most expressed laccase in developing seeds (see section 2.5.1), whereas a 40-cycle PCR resulted in detectable signals for all the samples (data not shown). According to Liang et al. (2006a), the weak expression of *AtLAC15* in roots observed by Cai and Wu (unpublished data), was confirmed by *Arabidopsis* microarray data (www.arabidopsis.org, no precise data set was cited). Present analysis of the transcriptomic data (see 2.1.1.2), found *TT10* expression only in developing siliques, seeds and pollen. However, this could be due to very strict cut-off values. Curiously, *TT10* signature tag in the massively parallel signature sequencing (MPSS, http://mpss.udel.edu/at/, Meyers et al., 2004), was found in only one out of two 21 day root samples, and moreover not in 24-48 hours post-fertilization siliques sample (McCaig et al., 2005). On the basis of these data, the presence of *TT10* in roots and leaves is questionable.

Reporter gene activity driven by the *TT10* promoter gave further support for the expression data. GUS activity, as previously reported by Pourcel et al. (2005), was mainly detected in the seeds, replum and transmitting tissue. Faint staining was also observed in the abscission zone of the petals and sepalns, but not in the petals and sepals themselves (see Fig. 2.7). Similar GUS pattern was also reported by Liang et al. (2006a), regarding siliques staining. However GUS activity was not reported in young siliques as well as precise information about the staining in the seed coat was missing.

To investigate potential stress-induced regulation of *TT10* gene expression, several experiments have been undertaken (see section 2.4). First analysis of publicly available transcriptomic data, as well as comments reported in the discussion by Pourcel et al. (2005), suggested that some stress conditions could modify *TT10* expression. Nevertheless, when strict cut-off and standard deviation filtering was used, *TT10* signal was below the detection level (discussed in Results 2.1.1.2 and 2.4). Moreover, in similar conditions as the ones described for the microarrays, transgenic seedlings carrying promoter fused to the reporter gene did not result in any detectable GUS staining. Surprisingly, in some cases blue staining was observed in the root tip and vitrified plantlets grown *in vitro*, but not in a reproducible manner. It would be possible that *TT10* could be expressed in the root tip, but in our experiments, proper combination of the developmental stage/treatment were not clearly identified to name the stress which could be involved. A possible induction could be due to oxidative or osmotic stress, because most of the experiments were carried out in the liquid medium and seem to be density of sowing dependent. The experiments carried out on the filter paper also resulted in blue staining of the root tip, which could be due to mechanical stress in this case.

Until now, *TT10* has been shown to be involved in flavonoid metabolism in seeds (Pourcel et al., 2005). Therefore it could also be involved in flavonol metabolism in roots, or other defense or detoxification-related process. Recently Liang et al. (2006b) have shown that *ZmLAC1* expression was induced in roots under salt stress conditions in seedlings grown in hydroponics. Moreover, several *Arabidopsis* laccase gene
transcript increased under PEG solution, which caused inhibition of root growth (Liang et al., 2006b). In our conditions we were able to detect induction of GUS activity driven by $TT10$ promoter neither in salt stress nor in seedlings grown with mannitol. Both PEG and mannitol treatments are commonly used to mimic osmotic stress conditions (Bouchabke-Coussa et al., 2008). It is possible that GUS activity is very weak and/or that when whole seedlings were floating in the culture solution, detectable gene induction due to stress conditions does not occur. Moreover stress response could be affected by sucrose which was always present in the growth media. It is possible that in Arabidopsis, other laccases but not $TT10$ are induced in roots in response to salt stress and/or osmotic stress.

The function of laccases is associated with defense against pathogen attack and wound healing (Mayer and Staples, 2002). However we were not able to detect GUS activity in wounded organs (leaves, stems). Previously, strong GUS activity was observed in aborted seeds (Pourcel et al., 2005). Trying to find a link between this phenomenon with stress response, we have emasculated Arabidopsis flowers which developed in seedless siliques in which strong GUS staining was observed in degenerated ovules (data not shown). It seems that activation of $TT10$ expression does not require either pollination or fertilization, which is surprising, because unfertilized ovules do not synthesize any PAs (Debeaujon et al., 2003). Most of our experiments suggest that $TT10$ expression is mainly under developmental regulation. Those results are in agreement with some previous studies that had shown that polyphenol oxidase (PPO) genes are mostly expressed in young developing tissues, with expression declining as development continues. Anyway, examples of strictly developmentally regulated as well as stress-induced PPO gene expression are well documented (Goldman et al., 1998; Constabel et al., 2000 and references therein). We could still consider that level of $TT10$ expression during seed and silique development could be stress-modulated. Nevertheless, it would be good to accompany studies of GUS induction by measurement of RNA accumulation, because elements required for stress response could be located outside the promoter, e.g. in the first intron (Rombauts et al., 2003; Rose et al., 2008).

It is important to add that some experiments aiming at comparing $TT10$ expression between various accessions were realized on silique samples collected from plants grown in the a growth chamber with controlled environmental conditions. In those samples, detected amounts of $TT10$ mRNA were comparable between accessions (data not shown). We could consider that the growth conditions in the culture chamber were more stable than those in the greenhouse, where temperature, light intensity and humidity variations could influence $TT10$ expression. It would be interesting to compare $TT10$ transcript accumulation in plants grown in various light intensities or photoperiods. We could hypothesize that response of different accessions is not the same and that those differences could be a result of plant adaptation to their habitats. Last but not least, it is important to note that all dissected promoter fragments studied in protoplasts resulted in detectable GUS activity. It may be only coincidence, but it could also indicate, that $TT10$ promoter contains cis-acting regulatory elements which are involved in stress response caused by protoplasting/wounding (Takeda et al., 2002). Interestingly, laccase activity was required for the cell-wall reconstruction in regenerating tobacco protoplasts (de Marco and Roubelakis-Angelakis, 1997).

### 4.1.3 Regulatory components

A promoter is a functionally defined 5’ part of the gene required for its transcription. It can be divided in the core part, which is required for assembly of the RNA polymerase II complex at the right position for directing basal level of transcription and the distal parts containing elements regulating spatio-temporal expression. Regulatory elements can also be located downstream, for instance in the first intron of the...
gene itself. It is not precisely defined and is species-dependent how far upstream or downstream regulatory elements and enhancers are located. Moreover, the three dimensional organization of DNA and chromatine structure constitutes and important part of gene regulation (reviewed in Rombauts et al., 2003).

The TT10 promoter studied by Pourcel et al. (2005) corresponds to a 2.0-kb region upstream from the translation initiation site, including 5' UTR. Interestingly, recently published results demonstrated that a 1.5-kb TT10 promoter fragment without 5' UTR was able to drive GUS expression in a tissue specific manner (Liang et al., 2006a). Moreover Liang et al. (2006a) considered 161bp upstream from the translation initiation site as 5'UTR, not the 34bp which were previously defined by RACE PCR (Pourcel et al., 2005). During this PhD, we wanted to precisely define a 'minimal' promoter containing all the elements sufficient for the spatio-temporal transcription of the gene in the seed coat. We were also interested in identifying transcription factors which could bind specific DNA sequences in the 'minimal' promoter. Promoter dissection and all the other results giving insight into the regulation of TT10 expression are discussed below.

4.1.3.1 Promoter and Cis-acting regulatory elements

Thanks to the 5' promoter dissection, a 194bp (between pTT10_3 and pTT10_2) region required for seed coat specific expression has been identified. The results of the promoter sub-dissection could further narrow down that region to 63bp (between pTT10_3 and pTT10_3C; see section 2.1.3.5). Moreover, site-directed mutagenesis suggests that the putative cis-acting regulatory element recognized as MYB2 binding site located in that region could be functional. This hypothesis is supported by the fact that several flavonoid biosynthetic genes are regulated by MYB genes (Debeaujon et al., 2003; Lepiniec et al., 2006) and another member of the laccase family has been recently demonstrated to be regulated by MYB58 and MYB63 transcription factors (Zhou et al., 2009). Results of site-directed mutagenesis suggested also that a DOF binding site for plant specific zinc finger transcription factors could be required for TT10 expression. Those are 4 bp motifs, associated with carbon metabolism genes and frequently found in the genome (Yanagisawa, 2000). In the TT10 promoter there are 5 DOF repeats within a 150 bp region, which seems to be more than average distribution of that motif in the Arabidopsis genome. In site-directed mutagenesis experiment, one of the substitutions was made in the DOF motif overlapping with I-box motif (see M4 on Fig. 2.6), which is a conserved sequence in promoters of light regulated genes (Terzaghi and Cashmore, 2003). It would be therefore interesting to further investigate the importance of both the DOF and I-box box binding motifs.

Realizing the promoter dissection experiment we have observed that the detectable GUS activity in seeds was abolished with 271bp (pTT10_2) promoter fragment, but faint staining was still detectable in the funiculus. Debeaujon et al. (2003) suggested that micropyle and chalaza zone could be particularly important to protect seed from pathogen invasion and to ensure moderate desiccation of the seed during maturation. Funiculus and seed abscission zone are in direct contact with chalaza, therefore it could be possible that to ensure proper protection of that zone, expression of the TT10 could be regulated differently in this area. The role of TT10 in this region as well as in the replum would rather be involvement in lignification of the dehiscence zones of the seed and silique. Moreover, this could be achieved in concert with other laccases and contribute to the pod shatter and seed dispersal (Liljegren et al., 2000).

It seems that element(s) required for high level of gene expression are localized upstream from the region required for tissue specificity. Existence of enhancer element(s), stabilizing the complex involved in the spatio-temporal regulation of gene expression would not be surprising (Rombauts et al., 2003). An alternative explanation would be that TT10 expression is regulated by two different mechanisms in the endothelium and outer integument layer 1. Analysis of GUS activity was mainly done by observation of
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the whole mount samples, therefore absence of GUS activity in endothelium would be masked by strong staining in the outer integument. Preliminary results of the GUS activity with the seed sections did not clearly answer this question (data not shown).

Liang et al. (2006a) showed GUS activity driven by the TT10 promoter without the 5'UTR. It suggests that the 5'UTR region of the promoter is not required for the GUS activity and that weak GUS activity observed for the constructs with pBI101-GTW vector, are rather vector-related. Similar drawback, with short promoter sequences was also observed for TT2 and TT16 (Christian Dubos, personal communication).

One more interesting aspect of the TT10 promoter could be discussed on the basis of the transient activity of all the promoter fragments studied in protoplasts. Even the shortest 171bp promoter fragment contained all the elements required for the GUS expression in this system. A possible explanation would be that protoplasting or the culture conditions are creating stressful conditions sufficient for ectopic GUS activity (de Marco and Roubelakis-Angelakis, 1997; Takeda et al., 2002). On the other hand, cells grown in the cell suspension are not differentiated and we could imagine that they miss negative regulator such as MYB4 or MYBL2 (Jin et al., 2000; Dubos et al., 2008) which are normally present in the plant.

![Diagram of TT10 promoter](image)

Figure 4.1: Organization of the regulatory elements in the TT10 Promoter.
Figure is summarizing promoter regions found to be required for seed, funiculus and strong expression of the TT10 gene. Asterixes (*) are highlighting the alternative 5'UTR and TATABOXES considered by Liang et al. (2006a).

It would be interesting to determine if the putative MYB2 binding site is functional and if an unidentified complex MYB/bHLH could regulate expression of TT10. One more indication for possible involvement of such a complex is in silico identified putative MYC2 binding motif around 150bp from MYB2 site. Abe et al. (2003) had found the combination of that motifs in many ABA responsive genes. Of interest is also the fact that the expression of several pathogen-related genes is upregulated in plants overexpressing MYC2/MYB2 (Abe et al., 2003). Taking into account that TT10 is a laccase involved in flavonoid biosynthesis, it is possible that one of the MYB family transcription factors is involved in its regulation. The reasons are multiple. Analysis of the phylogenetic relationship of the MYB TF involved in flavonoid biosynthesis shows that they form cluster (Stracke et al., 2001). Moreover, the genes regulating the early steps of the phenylpropanoid
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pathway (MYB75/PAP1, MYB90/PAP2) or flavonols (MYB11, MYB12, MYB111) are even more closely related and are forming clades in this cluster. We could expect that one of the MYBs in this cluster could be involved in regulation of TT10 expression. Interestingly, by co-expression analysis we have found MYB5 TF, which is also in this clade. Moreover, two other MYB TF, MYB58 and MYB63, have been recently shown to regulate expression of LAC4 (Zhou et al., 2009). Those MYBs cluster together, but not with the above-mentioned TF involved in the regulation of phenylpropanoid genes (Stracke et al., 2001). Based on all those data, we can make hypothesis that TT10 could be regulated by one of the MYB transcription factors. However, if it would be one more related to the MYBs involved in regulation of the flavonoid biosynthetic genes or one(s) regulating LAC4 is difficult to predict.

One more important point needs to be added to the study of the involvement of the MYB transcription factors in the regulation of the TT10 expression. Two mutants chosen from the co-expression studies, myb76 and myb99 showed decrease in TT10 transcript accumulation. It is possible that this slight decrease is not sufficient to observe some impact on flavonoids, because analysis of the flavonoid composition of mature seeds from those plants did not reveal any differences compared with wild-type. It might be that regulation of TT10 expression is ensured by several transcription factors with redundant function and therefore studies should consider multiple mutants in these regulatory genes. It would be interesting to check if TT10 expression could be further down-regulated in double/triple mutants of the MYBs being in the same clades with myb76 and myb99 (Stracke et al., 2001). It could be also interesting to see, if the GUS pattern driven by the TT10 promoter is modified in those mutants. Regarding the putative MYB binding sites found by in silico analysis, it could be also interesting to check if they could bind to the TT10 promoter.

4.1.3.2 Candidate transcription factors for developmental regulation

In silico analysis and promoter dissection studies were aiming at localizing regions in the upstream sequence of the TT10 gene which could contain cis-acting regulatory elements and together with the TATA box would constitute the promoter of the gene. In parallel, we have also looked for transcription factors which could be involved in the regulation of TT10 expression. Some results were already obtained by Pourcel et al. (2005), showing that TT10 is not regulated by the complex regulating BAN expression (Debeaujon et al., 2003; Baudry et al., 2004, 2006).

Results obtained during this work, are consistent with the preliminary data indicating that TT1, TT16 and TTG2 could regulate TT10 (Pourcel, 2006). In this case, the introduction of the promoter:GUS cassette into the regulatory mutant backgrounds was done by crossing, not by transformation (Pourcel, 2006), therefore any problem due to the influence of the insert position on promoter activity has been minimized. Moreover, the observation of GUS activity on seed sections revealed delayed TT10 promoter activity in tt1 and tt16 mutants. This could be explained by defects in endothelium development in the seeds of those plants (Sagasser et al., 2002; Nesi et al., 2002). A similar pattern of promoter activity was observed in the endothelium of the ttg2 mutant, which seems to be normally developed. However, the cells could be not properly differentiated (Johnson et al., 2002). The TTG2 gene has been also shown to be involved in mucilage accumulation and its promoter activity was reported to be active in the whole seed coat (Johnson et al., 2002). We have shown that it is only active in endothelium and mucilage layers. We could consider that TT10 expression is regulated by those genes in the endothelium. However, the observed modification of the GUS pattern could be an indirect effect. Promoter activity in the outer integument I layer could be regulated differentially from the one in endothelium, because the GUS activity in that layer seems to be not affected in tt1, tt16 and ttg2 mutants. It is possible that the regulation of gene expression in the seed
coat is very complex, e.g. regulation of the BAN gene in the endothelium seems to be different from that in micropyle and chalaza zones (Debeaujon et al., 2003). Taking in account that TT10 is also involved in flavonol metabolism (Pourcel et al., 2005), we could expect that its expression in the outer integument 1, is similar to the one of genes of that branch of the flavonoid pathway. Expression of TT10 was only slightly reduced in myb11, myb12, myb111 which are mutants in genes known to be required for flavonol biosynthesis in vegetative parts (Stracke et al., 2007) and seeds (Routaboul, unpublished results).

Current model for the regulation of PA biosynthesis in the seed coat involves TT2, TT8 and TTG1 which are required for the BAN expression for example. Moreover, TTG2 seems to act downstream from TTG1, whereas TT16 upstream from TT2, and TT1 seems to be necessary for BAN expression in a few cells at the endothelium base. Also fertilization is required for PA accumulation in general (Debeaujon et al., 2003). Taking in account that TT10 expression is not modified in tt2, tt8, and ttg1 mutants and only slightly modified in tt1, tt16 and ttg2, we should rather expect distinct regulators.

Interestingly, Arabidopsis lacks polyphenol oxidases of the catechol oxidase (CO) type (Pourcel et al., 2005). Therefore we may hypothesize that laccase such as TT10 has been recruited evolutionary to compensate for the lack of CO. This is relevant with the fact that both TT10 and COs have flavonoids as substrates. TT10 is not directly involved in the biosynthesis of flavonoids, but only in the oxidative polymerization of the final products (Pourcel et al., 2005), therefore regulation of its expression could evolve as a special function of one of the laccase family genes in lack of the catechol oxidases in Arabidopsis (Constabel et al., 2000; Thipyapong et al., 2004; Pourcel et al., 2005). It would be interesting to find out if the TT10 function could be replaced by other laccases as well as a catechol oxidases from other species.

4.1.3.3 Candidate transcription factors for stress regulation

Previously discussed potential stress regulation of TT10 led us to study of transcript accumulation in mutants in genes involved in pathogen and stress response (see section 2.2.5). We did not observe any significant differences in the amount of TT10 mRNA in any of the mutants considered, except npr1-1. Surprisingly, transcript accumulation in npr1-2 allele was comparable to the wild type. However both alleles studied come from the EMS mutagenesis and in both of them protein is detected (Cao et al., 1997; Somssich, 2003). Moreover, it has been shown that npr1-1 mutant was non-functional with respect to systemic acquired resistance, but in an NPR1-GFP overexpressor line carrying npr1-1 mutation, the npr1-1 protein was part of the detectable oligomeric complex. The npr1-2 mutation resulted in a cysteine to tyrosine conversion and is thought to result in a non-functional protein (summarized in Somssich, 2003). NPR1 function is well studied and it is thought to play an important role in the cross-talk between salicylic and jasmonic acid-dependent defense pathways (Somssich, 2003; Dong, 2004; Pieterse and Loon, 2004). It would be possible that the mutation in the npr1-1 line resulted in inactivation of the protein domain required for recruitment of factors involved in TT10 expression. We have found that TT10 expression was also decreased in npr1-5 allele. However recently we realized that this mutant contains a T-DNA insertion carrying kanamycin resistance gene and the PR-1α:tm2 reporter gene and is in Nossen background. Taking into account the natural variation occurring for TT10 gene expression, we were not able to conclude on that result. It would be necessary to check other alleles of the npr1 mutant. It might be also interesting to include mutants in TGA proteins which belong to bZIP family of transcription factors. These TFs has been shown to interact with NPR1 (reviewed in Pieterse and Loon (2004) and Dong, 2004).

We have found several putative W-boxes in the TT10 promoter sequence, which are binding sites for WRKY TFs. This family of TF is involved in regulation of plant defense processes (Pandey and Somssich, 2009). Moreover, elicitor-induced expression of transcription factors and metabolic reprogramming of
secondary metabolism have been demonstrated in *Medicago truncatula* cell suspension, in which WRKY transcription factors expression were one of the most affected by yeast elicitor and methyl jasmonate treatment. Several laccases have been shown to be induced by that treatment (Naoumkina et al., 2008). Interestingly a laccase has recently been proposed as a candidate gene to explain a QTL for resistance to nematode in soybean (Iqbal et al., 2008). Other recent report demonstrated that knocking down expression of *WRKY23* resulted in lower infection of the cyst nematode *Heterodera schachtii* (Grunewald et al., 2008). Moreover plants overexpressing *WRKY23* were accumulating more quercetin-3-O-rhamnoside (Godelieve Gheysen, personal communication). Following the indications that laccase could be involved in nematode resistance, and that *WRKY23* was shown to mediate infection rate of the cyst nematode we were interested in possible relation with *TT10*. Analysis of the transcript accumulation revealed, that *TT10* expression was not modified in three alleles of the *wrky23* studied. We could assume that *WRKY23* is not involved in regulation of *TT10* expression. However all the lines studied had T-DNA insertion in the promoter region of the gene and they are not knockouts (Grunewald et al., 2008). It is possible that even low expression was sufficient to sustain *TT10* transcription at the wild-type level.

Studies of *TT10* expression in other mutants affected in plant defense genes did not reveal any differences in the transcript accumulation. However, all our experiments concerned not stressed plant. We could expect that in those conditions plants would have some basic level of pathogen related genes, which expression is only induced in response to environmental challenges. Induction of the defense genes would be part of plant plasticity and adaptation strategy, therefore it would be interesting to test how expression of *TT10* and flavonoid biosynthetic genes is maintained in those mutants under various stresses (e.g. in response to pathogen attack, dehydration, different light intensities).

Last, during our quest to find regulator of *TT10* expression, we have observed that the *jar1* mutant which is affected in jasmonic acid perception (Lorenzo et al., 2004), was accumulating less quercetin-3-O-rhamnoside and biflavonols, which could suggest some involvement in the biosynthesis of those flavonols. Interestingly, *jar1* is located in the interval found in the QTL mapping for the flavonoid composition in mature seeds (Jean-Marc Routaboul, unpublished results).

### 4.1.3.4 What have we learned from natural variation?

Study of natural variation is a novel approach to discover new functions especially those involved in adaptation to a specific habitat. Phenotypic differences are often due to allelic variations at several loci and the contribution of each locus to the phenotype can be quite small (Weigel and Nordborg, 2005). It has been previously shown that single nucleotide polymorphism between gene alleles could result in differential gene expression or protein activity (de Meaux et al., 2005; Bentsink et al., 2006; Loudet et al., 2007). Here we wanted to find out if level of *TT10* expression, which varies between accession, could be attributed to polymorphism in *cis*-acting regulatory elements in the promoter. We have found several SNPs in *Arabidopsis* accessions studied, but we were not able to link them with the level of transcript accumulation, nor flavonoid composition of matures seeds.

Comparison of long promoter sequences between Col-0 (2.2-kb) and Ler (1.5-kb) revealed several indels and more than 700bp deletion in Ler accession (Ishihara, 2007). Interestingly we have found that ATCOPIA42 transposon identified in *TT10* promoter by FLAGdb++ server (Samson et al., 2004), may correspond to this large deletion (see Appendix, Fig. 5.7). It seems that, in Ler, the transposon was excised or inserted in Col-0. We were not able to identify the remaining LTR sequences. It is important to note that the transposon is located in the region which was found to be required for high *GUS* expression. Interestingly the transposon region in the *TT10* promoter is methylated (see section 4.1.3.7, Chromatine structure), suggesting that it may be inactive. Indeed in plants, silent transposable elements are methylated.
4.1. REGULATION OF TT10 GENE EXPRESSION

(Rabinowicz et al., 2003). It is difficult to predict which effect transposons can have on gene expression. Both situations were reported, one with which transposon promoters can activate neighboring gene expression and act as an enhancer, and the other with which they can also act as insulators, and result in a decrease or complete block of gene expression (Dorsett, 1999; Valenzuela and Kamakaka, 2006). It would be interesting to check what is the effect of the transposon present in TT10 promoter on its expression.

The study of the TT10 Col-0 promoter in other accessions backgrounds (see section 2.3.5) revealed that tissue specific activity was not altered. Moreover, fragments studied in the promoter dissection experiment (see section 2.1.3.1) pointed at the same region being required for the seed coat activity of the promoter in Col-0 and Ws-4. Interestingly also the upstream element required for high GUS activity in Col-0 background is also required for strong GUS induction in Ws-4. Those results suggest that the differences in the trans-regulation (i.e. due to transcription factor) are not likely because cis-acting regulatory elements in the promoter sequence from Col-0 accessions are recognized by transcription factors in other accessions and result in the same tissue specific promoter activity.

4.1.3.5 Transcription factor library screening

In case of yeast one- and two- hybrid screening auto-activation is a technical problem encountered with some promoters and proteins. Very often it can be overcome by adding 3-AT, the competitive inhibitor of the HIS3 product. However in small scale experiments, the concentration could be raised up to 25-50mM (Baudry et al., 2004; Xiao, 2006). For screening of the REGIA transcription factor library it was not advised to use more than 5mM 3-AT because of the the sensitivity of the yeast and lack of detection of weak interactions. We have tried to use smaller fragments of the TT10 promoter, but this strategy did not eliminate the auto-activation. It would be good to consider even smaller promoter fragments e.g. 63bp (region between pTT10_3 and pTT10_3C) promoter fragment containing the MYB2 binding site or even isolated motifs identified in the site-directed mutagenesis experiment. Another solution could be use of another MATα yeast strain which could enable to study longer promoter fragments (Xiao, 2006).

Yeast one-hybrid screen, could give an indication of the class/family of the transcription factors which could be involved in regulation of the target gene expression. However later on their functionality should be confirmed in planta. In some cases, interaction found in yeast, would not be possible in planta, because of differential spatio-temporal presence of the transcription factor and expression of the gene considered in the experiment.

4.1.3.6 Co-expressed genes

We have analyzed TT10 expression and flavonoid composition of mature seeds of several mutants chosen on the basis of co-expression analysis. Apart from myb76 and myb99, all the other mutants analyzed accumulated wild-type amount of TT10 mRNA and flavonoids. However other group of genes co-expressed with TT10 during plant development have been proposed and should be more relevant, because they are co-expressed during seed development (see section 2.2.3.3). Interestingly, among those new candidates was MYB5, which may be the most promising candidate to study. The relevance of the other genes should be considered based on the literature studies.

It is important to keep in mind that co-expressed genes are not necessarily involved in regulation of TT10 expression. It is possible that TT10 and co-expressed genes are regulated by the same factor. To test this hypothesis it would be required to compare their promoter sequences for presence of the common cis-acting regulatory elements. On the other hand, the list of the co-expressed genes miss those which are expressed in several tissues.
4.1.3.7 Epigenetics?

Recently small RNAs (smRNAs) have been recognized as one of the players for regulation of gene expression in plants. Depending on the formation process, smRNAs can be divided in several classes as recently reviewed by Voinnet (2009). smRNAs regulate gene expression in a process called post-transcriptional gene silencing (PTGS), RNA silencing or RNA interference (RNAi). Many plant responses related to adaptation to changing environment have been shown to be regulated by smRNAs (Voinnet, 2008). Interestingly, several laccases have been shown to be predicted targets of \( \text{miR}397 \) (LAC2, LAC4 and LAC17), \( \text{miR}408 \) (LAC3, LAC12 and LAC13) and \( \text{miR}857 \) (LAC7) (Jones-Rhoades et al., 2006), which are induced in seedling grown in hydroponics conditions with copper-depleted medium (Abdel-Ghany and Pilon, 2008). Those \text{miRNAs} are thought to be involved in the regulation of expression of non essential copper proteins (Abdel-Ghany and Pilon, 2008). There are no predicted \text{miRs} for LAC15. Surprisingly Abdel-Ghany and Pilon (2008) reported that LAC15 was ubiquitously expressed and its transcript level was moderately affected by copper availability in the growth medium. Moreover, it was observed that LAC15 expression was 5-100-fold higher (depending on the plant organ tested) in plants grown with high copper concentration compared to the no copper condition (Abdel-Ghany and Pilon, 2008). It is possible, that in the plants grown in the hydroponic conditions with various concentration of copper available, \( TT10 \) expression could be induced. However, the only organs in which \( TT10 \) expression was assessed were leaf, stem, root and flower. In all cases expression was very low in range of 100-10000-fold lower that the reference gene actin (Abdel-Ghany and Pilon, 2008). We could question the significance of those results because of very low level of \( TT10 \) expression in those experiments. Moreover no miRNA was predicted to target \( TT10 \). On the other hand it could explain that in our conditions, plants grown in soil had limited availability of copper and therefore we were unable to detect \( TT10 \) transcript in tissues other than siliques.

Promoters are often regarded as linear stretches of DNA, whereas regulation of gene expression is enabled by interaction of all the components in the three dimensional structure. DNA is packed into chromatin and often access to the DNA template for transcription is limited. Moreover DNA methylation has been shown to repress transcription initiation (Rombauts et al., 2003). We checked publicly available data ([http://epigenomics.mcdb.ucla.edu/](http://epigenomics.mcdb.ucla.edu/), UCSC Genome Bioinformatics) for the DNA and histone methylation of the \( TT10 \) gene and promoter. These data revealed two methylated regions in the promoter and histone H3K27m3 methylation at the beginning of the fifth exon and on the border between the fifth exon and fifth intron. The experiments were carried out with whole seedlings (10-14 days) or aerial part of 5-week-old plants (Zhang et al., 2006, 2007). For the map of methylated regions see Appendix, Fig. 5.6.

Interestingly, recent work has shown that a high level of \( \approx 24 \) nt small interfering RNA is present in Ler accession compared to Col-0. These siRNAs were directing DNA methylation and heterochromatinization at the \( hAT \) transposon element adjacent to the promoter of FLOWERING LOCUS C gene in Ler, whereas the same element was not affected in Col-0 (Zhai, 2008). Methylation could be one of the mechanisms to switch off \( TT10 \) expression in plant organs other than siliques and seeds or affect the level of its expression in various accessions.
# 4.2 TT10 and flavonoid metabolism in seeds

## 4.2.1 TT10 and flavonoid metabolism

Pourcel et al. (2005) have demonstrated that TT10 is involved in oxidative polymerization of flavonols and tannins. During this work we analyzed the correlation between TT10 expression levels and the amount of soluble tannins as well as accumulation of quercetin-3-O-rhamnoside (Q-3-O-R) and biflavonols. For that reason Ler accession has been transformed with Col-0 genomic sequence to increase the amount of TT10.

We have achieved not only to bring the TT10 transcript level to the one observed in Col-0, but we also observed until 40-fold higher expression in one line. Normally the soluble fraction of PAs is regarded as not or slightly oxidized and not cell-wall nor protein-bound, whereas after oxidation they are regarded as insoluble/non extractable. By overexpressing TT10, we were expecting to increase oxidation and to extract less soluble PAs from the 'complemented' lines. Visual inspection of the seeds revealed that they were darker than the Ler wild-type control, but surprisingly none of the lines accumulated less soluble PAs. Interestingly, four transgenic lines accumulated less Q-3-O-R, but expected increase in biflavonol amount was not observed. The results obtained are puzzling and difficult to interpret. Moreover increase of the transcript accumulation may not always be correlated with the amount of active protein, therefore further comments are only hypothetical.

The most important fact was that in none of the lines studied a decrease of soluble PA fraction was observed. We could hypothesize that the wild-type TT10 expression is sufficient to metabolize tannins available in Ler and for some unknown reason oxidation can not go beyond that limit. Indeed, TT10 has been shown to trigger oxidative polymerization of tannins and flavonols, but subsequent reactions on which color and probably the extractability of the oxidized phenols depends are still not well studied (Nicolas et al., 1993; Pourcel et al., 2005). It is highly probable that not only oxidation but also subsequent steps leading to formation of the insoluble fractions of PAs are driven enzymatically. However, this process could also be spontaneous due to the highly chemically reactive nature of intermediate quinones.

Because of the natural variation observed for TT10 expression we were also interested in the effect of mutation in TT10 gene in other Arabidopsis accessions. Those studies clearly confirm the involvement of TT10 in oxidation of tannins and flavonols, because all the alleles of tt10 mutant accumulated more soluble tannins and quercetin-3-O-rhamnoside (Q-3-O-R) but less biflavonols than respective wild-type plants.

Interestingly Cvi, normally accumulates much more tannins than other accessions, but almost no Q-3-O-R and biflavonols. As expected the tt10-7 mutant allele (in Cvi background) accumulates more soluble PAs and Q-3-O-R like other tt10 mutant alleles. Regarding low level of biflavonols in the Cvi, one would expect that Q-3-O-R are not synthesized. However, detection of Q-3-O-R in the tt10-7 mutant suggests that other enzyme or other unknown feature would be required to act in concert with TT10 to synthesize biflavonols. It is also possible that in Cvi, biflavonols are further metabolized what would explain that they are detectable in very low amount in this accession.

It is clear that TT10 is involved in tannin and flavonol oxidation, however it seems that some differences between accessions could have arisen in respect to the amount and classes of the flavonoids accumulated (Pourcel et al., 2005; Routaboul et al., 2006 Jean-Marc Routaboul, unpublished results). There are still many aspects of flavonoid metabolism to be discovered to fully understand their accumulation and oxidation.

## 4.2.2 Other functions?

Liang et al. (2006a) reported the involvement of TT10 in lignin metabolism in seeds and root elongation. It is possible that TT10 is involved in the lignification not only in the seeds, but also in the replum where its promoter activity was observed. It is also possible that in certain conditions TT10 could be involved in
flavonol metabolism in roots. However, until now those functions have not been demonstrated.

4.3 Regulation in the multi-gene family

It seems that laccases have acquired their specific functions very early during evolution and they are involved in resistance to various stresses (Mayer and Staples, 2002; Thipyapong et al., 2004). One of the functions related to plant defense would be oxidation of flavonoids (Pourcel et al., 2005). Interestingly in *Arabidopsis* genome no typical catechol oxidases were found (Pourcel et al., 2005, 2007). Laccases have a broad spectrum of substrates and they can oxidize ortho- and para-diphenols. Therefore, they could be involved in various aspects of phenylpropanoid metabolism (Mayer and Staples, 2002; Pourcel et al., 2007). Pourcel et al. (2005) have proposed that in absence of catechol oxidase, laccases may have evolved to fulfill this lack (i.e. catalyzing o-diphenol oxidation).

Interestingly, in this study we showed that **TT10** is the only member of the family expressed in seeds. This fact answers the question why a single mutant in this laccase gene results in a clear phenotype. It seems that **TT10** is a laccase specialized in flavonoid oxidation by co-localizing with the substrates in plant tissues.

Strong GUS staining driven by **TT10** promoter in aborted seeds was previously associated with senescence/cell death (Pourcel et al., 2005). Interestingly, during the characterization of expression profiles and GUS activity driven by promoters of other laccases, we have observed that the **LAC5** promoter resulted in strong GUS activity in degenerated ovules and aborted seeds. It seems that in such a case of developmental defect, **TT10** together with **LAC5** could be involved in protection of a fragile zone caused by cell death. Analysis of the transcriptomic data suggests that **LAC5** is highly induced during senescence (data not shown) and profile of GUS activity in aborted seeds observed for **TT10** and **LAC5** highly resembles that of **SENESCENCE ASSOCIATED GENE 12** promoter (data not shown).

It is possible, that in certain conditions several laccases act in concert to ensure sufficient protection of plant. Other example of overlapping expression of several laccases would be silique replum in which in addition to **TT10**, GUS activity driven by the promoters of **LAC5**, **LAC12** (this study), **LAC4** (data not shown) and **LAC2** (Serge Berthet, unpublished results) were also detected. This function of the laccase in various organs could overlap and therefore studies of single mutants would not result in identification of significant phenotypes, with **TT10** being an exception.

4.4 Final remarks

We should acknowledge the involvement of the **TT10** in plant protection against various stresses/environmental challenges. It would rather be a preformed defense by preparation of the oxidized flavonoid shield - seed coat - to protect the embryo, than direct response to pathogen attack. Regulation of the **TT10** expression and importance of flavonoid oxidation still needs to be further investigated. Moreover studies of other members of laccase family will bring interesting information about evolutionary specialization of gene function by tissue specific gene expression.
Chapter 5

Appendices

5.1  

*In silico* analysis of the *TT10* promoter

*TT10* (Col-0) promoter sequence used in this study:

cacactgttttgcctggaatgcccaagcaacaaatcttttcactatatctttatatataatattttttacaagaattct
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<td>CACFTTPPCA1 209 (+) YACT</td>
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<td>DOFCOREZM 209 (-) AAGAGA</td>
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</table>
ANALYSIS OF THE TT10 PROMOTER
**5.1. IN SILICO ANALYSIS OF THE TT10 PROMOTER**

The list of putative cis-acting regulatory elements had been obtained by querying *AtcisDB Search Engine* (Molina and Grotewold, 2005) for locus ID At5g48100 (up dated 16.04.2009). The image of the genomic region of the At5g48100 locus is a visualization of the data presented in the table (adapted from AtcisDB).

**Table 5.1: Putative cis-acting regulatory elements found in TT10 promoter with Signal Scan.**

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<th>Element Name</th>
<th>Query Strand</th>
<th>Match Strand</th>
<th>Element ID</th>
<th>Match Score</th>
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<td>+</td>
<td>GTG710</td>
<td>(+) TGCA</td>
</tr>
<tr>
<td>Metacore ZM</td>
<td>+</td>
<td>+</td>
<td>DOFCOREZM</td>
<td>(+) AAAG</td>
</tr>
<tr>
<td>WBOXPCWRKY1</td>
<td>+</td>
<td>+</td>
<td>POLLEN1LELAT52</td>
<td>(+) AGAAA</td>
</tr>
<tr>
<td>ELRECOREPCHR1</td>
<td>+</td>
<td>+</td>
<td>DRECOREZM</td>
<td>(+) AAAG</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>DRECOREATSLTR1</td>
<td>(-) GAGAC</td>
</tr>
<tr>
<td>WBOXINTERF3</td>
<td>+</td>
<td>+</td>
<td>TATAAT</td>
<td>(+) TATAAT</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>TATABOX4</td>
<td>(-) TATAAA</td>
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<tr>
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<td>+</td>
<td>TATAPVRNALLEU</td>
<td>(-) TTTATA</td>
</tr>
<tr>
<td>GTGANT10</td>
<td>+</td>
<td>+</td>
<td>LECPLEACS2</td>
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<tr>
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<td>+</td>
<td>ROOTMOLIFTAP</td>
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<td>+</td>
<td>SORLREP3AT</td>
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<td>+</td>
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<td>(+) TATCT</td>
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<td>DRECOREZM</td>
<td>(-) AAAG</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
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<td>(+) CTCTT</td>
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**5.1.2 CAREs found in AtcisDB**

![AtcisDB Search Engine](image)

Figure 5.1: Visualization of cis-acting regulatory elements found with AtcisDB search engine. Blue lines represent chromosome 5 and At5g48100/TT10 gene - black box. TT10 is transcribed from negative strand and At5g48090 from positive strand - yellow box. Bottom part of the figure is focusing on the promoter sequence analyzed. Grey boxes are representing putative cis-acting regulatory elements identified and listed in the Table 5.2.
### Table 1.2

<table>
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<tr>
<th>Binding Site (BS) Name</th>
<th>BS Genome Start</th>
<th>BS Genome End</th>
<th>Binding Site Sequence</th>
<th>Binding Site Family/TF</th>
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<td>AtMYC2 BS in RD22</td>
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<td>19510315</td>
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<td>19510146</td>
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<td>19512409</td>
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<td>19512752</td>
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5.2 Primers

All the primers were purchased from SIGMA. They were desalted, except primers used for the site-directed mutagenesis which were PAGE-purified. Primers were delivered lyophilized and dissolved in water after arrival to obtain 100μM stock solution.

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<th>Name</th>
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<th>Orientation on promoter</th>
<th>Restriction site</th>
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Table 5.3: Primers used for promoter dissection to clone fragments in pBS-GUS vector.

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<tr>
<td>pTT10_ -4.5kb</td>
<td>cagcatgtattttctctctatg</td>
<td>F</td>
</tr>
<tr>
<td>pTT10_ -4.0kb</td>
<td>taaaccgtgatttttcctgc</td>
<td>F</td>
</tr>
<tr>
<td>pTT10_ -3.5kb</td>
<td>tttaaccttataaactcaacct</td>
<td>F</td>
</tr>
<tr>
<td>pTT10_ -3.0kb</td>
<td>tttaaccatgtgatgtgcatg</td>
<td>F</td>
</tr>
<tr>
<td>pTT10_ -2.5kb</td>
<td>acaggctgtaagaagactgtg</td>
<td>F</td>
</tr>
<tr>
<td>pTT10_ 0.0kb</td>
<td>ataaattgaagaagaggtgca</td>
<td>R</td>
</tr>
</tbody>
</table>

Table 5.4: Primers used for amplification of long promoter fragments in Ler and Cvi accessions.
<table>
<thead>
<tr>
<th>Name</th>
<th>5’-3’ sequence</th>
<th>Orientation on promoter</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTT10_3 SmaI</td>
<td>gtaCCCGGGattaacacaaaaacgtaacgag</td>
<td>F</td>
<td>SmaI</td>
</tr>
<tr>
<td>pTT10_2 SmaI</td>
<td>gtaCCCGGGatagttacggaacgttaacgag</td>
<td>F</td>
<td>SmaI</td>
</tr>
<tr>
<td>pTT10_2 SacII</td>
<td>gtaCCCGGGagttaacggaacagcataaatgcg</td>
<td>R</td>
<td>SacII</td>
</tr>
<tr>
<td>pTT10_1 SacII</td>
<td>gtaCCCGGGacaaatatgttttccgtaacgc</td>
<td>R</td>
<td>SacII</td>
</tr>
<tr>
<td>pTT10 0.0 kb SacII</td>
<td>gtaCCCGGGaagagttttagtaaatattacc</td>
<td>R</td>
<td>SacII</td>
</tr>
</tbody>
</table>

Table 5.5: Primers used to amplify promoter fragments for transcription factor library screening with pHISi vector.

<table>
<thead>
<tr>
<th>Name of the construct and primers used</th>
<th>5’-3’ sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1 F</td>
<td>CTAATTTACACAAAAATTTAAGCTTACGAATA</td>
</tr>
<tr>
<td>M1 R</td>
<td>TATCCGAAACTCGTTAAAAATTTGTGTTTAG</td>
</tr>
<tr>
<td>M2 F</td>
<td>GTTCCGGAATTTTTCTCTATCATTTATATAATAAAAGG</td>
</tr>
<tr>
<td>M2 R</td>
<td>CCTTTATTATTATAATGGATAGCAGAAAAATTTCGGAAC</td>
</tr>
<tr>
<td>M3 F</td>
<td>GCTGCGCTACATTATTTTCAGAAAGTTGATTG</td>
</tr>
<tr>
<td>M3 R</td>
<td>AACATTTGATTTTGAATCTAGGGAACCAAG</td>
</tr>
<tr>
<td>M4 F</td>
<td>GTGATTTTTTTTTTTTATTGAAAAATTTTTGACTAGTTGATTGATGTC</td>
</tr>
<tr>
<td>M4 R</td>
<td>GACATCAATCAACTAGTTCAAAAAATTTATATAAAATATAC</td>
</tr>
<tr>
<td>M5 F</td>
<td>GAACATATTGGATTGATGATATAAAACTTTACTATATAG</td>
</tr>
<tr>
<td>M5 R</td>
<td>CATATATAGTAAGGTCTTATCATCAATCAACTAGTTC</td>
</tr>
</tbody>
</table>

Table 5.6: Primers used for site-directed mutagenesis on pTT10_3 promoter fragment in pBS-GUS vector.

<table>
<thead>
<tr>
<th>Name</th>
<th>5’-3’ sequence</th>
<th>Orientation on promoter</th>
<th>Probe for:</th>
<th>Probe size</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDN-ATG0</td>
<td>gcttaccgaaacatattttgtaa</td>
<td>F</td>
<td>5’ region</td>
<td>820bp</td>
</tr>
<tr>
<td>MDN-Rev 800 TT10</td>
<td>caaaatattgctttgcttg</td>
<td>R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JFW-TT10</td>
<td>gtataaaaaatacatgaccctgcttgg</td>
<td>F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDN-rv-0-long</td>
<td>gttgacaagttatacttttgttatactgg</td>
<td>R</td>
<td>3’ region</td>
<td>990bp</td>
</tr>
</tbody>
</table>

Table 5.7: Primers used for probes preparation for BAC library screening.

<table>
<thead>
<tr>
<th>Name</th>
<th>5’-3’ sequence</th>
<th>Orientation on promoter</th>
<th>GATEWAY recombination site</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTT10_3</td>
<td>attaacacaaaaacgtaacgag</td>
<td>F</td>
<td>attB1</td>
</tr>
<tr>
<td>pTT10_3C</td>
<td>aataaaagtgtaatgctgctgccc</td>
<td>F</td>
<td>attB1</td>
</tr>
<tr>
<td>pTT10_3B</td>
<td>aactatctgatctgtgctgcag</td>
<td>F</td>
<td>attB1</td>
</tr>
<tr>
<td>pTT10_3A</td>
<td>tactatatcatttataactatgtg</td>
<td>F</td>
<td>attB1</td>
</tr>
<tr>
<td>pTT10_2</td>
<td>atacccagcttctgtttcctgg</td>
<td>F</td>
<td>attB1</td>
</tr>
<tr>
<td>LAC15_R1</td>
<td>tttggaagagtttttactatatattacc</td>
<td>R</td>
<td>attB2</td>
</tr>
</tbody>
</table>

Table 5.8: Primers used for promoter dissection - GATEWAY® cloning.
### 5.2. PRIMERS

<table>
<thead>
<tr>
<th>Name</th>
<th>5′-3′ sequence</th>
<th>Comments</th>
<th>AGI</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAC1 5GST</td>
<td>TTCCGTAACACACGCCG</td>
<td>CATMA GST</td>
<td>At1g18140</td>
</tr>
<tr>
<td>LAC1 3GST</td>
<td>AGAATCTCTTCTCGCTTACC</td>
<td>CATMA GST</td>
<td>At2g29130</td>
</tr>
<tr>
<td>LAC2 5GST</td>
<td>CATATCCAGAAAGGAAAGGGG</td>
<td>CATMA GST</td>
<td>At2g30210</td>
</tr>
<tr>
<td>LAC2 3GST</td>
<td>CTGGATACCTTCTCGCTTACC</td>
<td>CATMA GST</td>
<td>At2g38080</td>
</tr>
<tr>
<td>LAC3 5GST</td>
<td>TGTGTGGCTGATGACATTG</td>
<td>CATMA GST</td>
<td>At2g40370</td>
</tr>
<tr>
<td>LAC3 3GST</td>
<td>CATTCCAAGAAAGGGAAAAAGG</td>
<td>CATMA GST</td>
<td>At2g46570</td>
</tr>
<tr>
<td>LAC4 5GST</td>
<td>GTGGTCAAATCGCTCCTGTT</td>
<td>CATMA GST</td>
<td>At3g09220</td>
</tr>
<tr>
<td>LAC4 3GST</td>
<td>TTGTGAAGATTCCTGGGGTG</td>
<td>CATMA GST</td>
<td>At5g01040</td>
</tr>
<tr>
<td>LAC5 5GST</td>
<td>GGTTTGGTTCATGACATTGTCACTT</td>
<td>CATMA GST</td>
<td>At5g01190</td>
</tr>
<tr>
<td>LAC5 3GST</td>
<td>GCACGCATTAAATCACCACTC</td>
<td>CATMA GST</td>
<td>At5g03260</td>
</tr>
<tr>
<td>LAC6 5GST</td>
<td>TTCCGTAAACACACGGCG</td>
<td>CATMA GST</td>
<td>At5g07130</td>
</tr>
<tr>
<td>LAC6 3GST</td>
<td>AGAATCTCTTCCCTGCGTAACCL</td>
<td>CATMA GST</td>
<td>At5g09360</td>
</tr>
<tr>
<td>LAC7 5GST</td>
<td>TGTGTGGCTGATGACATTG</td>
<td>CATMA GST</td>
<td>At5g09360</td>
</tr>
<tr>
<td>LAC7 3GST</td>
<td>CATTCCAAGAAAGGGAAAAAGG</td>
<td>CATMA GST</td>
<td>At5g09360</td>
</tr>
<tr>
<td>LAC8 F</td>
<td>ATGGGTTGTCCTCAGATTTATCGCTAATA</td>
<td>CATMA GST</td>
<td>At5g48100</td>
</tr>
<tr>
<td>LAC8 R</td>
<td>TGTTCTCTCGTACAAATCAGTTCAAGCATT</td>
<td>CATMA GST</td>
<td>At5g48100</td>
</tr>
<tr>
<td>LAC9 F</td>
<td>ATGGGTTGTCCTCAGATTTATCGCTAATA</td>
<td>CATMA GST</td>
<td>At5g58910</td>
</tr>
<tr>
<td>LAC9 R</td>
<td>GTGTAGAGAAATGGAAGCATGGTCGTAAA</td>
<td>CATMA GST</td>
<td>At5g60020</td>
</tr>
<tr>
<td>LAC10 5GST</td>
<td>ATTAAGCCGGGTCACAGCTAC</td>
<td>CATMA GST</td>
<td>At5g09360</td>
</tr>
<tr>
<td>LAC10 3GST</td>
<td>GTATGATGACTTCTTCTCTATGTG</td>
<td>CATMA GST</td>
<td>At5g09360</td>
</tr>
<tr>
<td>LAC11 5GST</td>
<td>TGAGATGGGAACACTACTCTGAT</td>
<td>CATMA GST</td>
<td>At5g09360</td>
</tr>
<tr>
<td>LAC11 3GST</td>
<td>CATTTCTTATGCAATTTCGGGG</td>
<td>CATMA GST</td>
<td>At5g09360</td>
</tr>
<tr>
<td>LAC12 5GST</td>
<td>CCAACCCCCAAAACGCTATTAC</td>
<td>CATMA GST</td>
<td>At5g09360</td>
</tr>
<tr>
<td>LAC12 3GST</td>
<td>CCTTGGCACCGGCTTTTAG</td>
<td>CATMA GST</td>
<td>At5g09360</td>
</tr>
<tr>
<td>LAC13 5GST</td>
<td>TGGCCTGAGCAATCTCACAG</td>
<td>CATMA GST</td>
<td>At5g09360</td>
</tr>
<tr>
<td>LAC13 3GST</td>
<td>TGGCCTGAGCAATCTCACAG</td>
<td>CATMA GST</td>
<td>At5g09360</td>
</tr>
<tr>
<td>LAC14 5GST</td>
<td>TTCACCGACCGGCTTTTAG</td>
<td>CATMA GST</td>
<td>At5g09360</td>
</tr>
<tr>
<td>LAC14 3GST</td>
<td>CCAACCCCCAAAACGCTATTAC</td>
<td>CATMA GST</td>
<td>At5g09360</td>
</tr>
<tr>
<td>LAC15 5GST</td>
<td>GATGTGAGAAATGGAAGCATGGTCGTAAA</td>
<td>CATMA GST</td>
<td>At5g09360</td>
</tr>
<tr>
<td>LAC15 3GST</td>
<td>CGGATTAAACCGATTCTACC</td>
<td>CATMA GST</td>
<td>At5g09360</td>
</tr>
<tr>
<td>LAC16 5GST</td>
<td>CCAACCCCCAAAACGCTATTAC</td>
<td>CATMA GST</td>
<td>At5g09360</td>
</tr>
<tr>
<td>LAC16 3GST</td>
<td>CATATCCAGAAAGGAAAGGGG</td>
<td>CATMA GST</td>
<td>At5g09360</td>
</tr>
<tr>
<td>LAC17 5GST</td>
<td>GTCTCTTCTTTCTTTACAAGCTTGCG</td>
<td>CATMA GST</td>
<td>At5g09360</td>
</tr>
<tr>
<td>LAC17 3GST</td>
<td>TGGCCTGAGCAATCTCACAG</td>
<td>CATMA GST</td>
<td>At5g09360</td>
</tr>
</tbody>
</table>

**Table 5.9:** Primers used to study laccase family genes expression.
CATMA GST primers are coming from the CATMA project: [http://www.catma.org/database/](http://www.catma.org/database/)

### Table 5.10: Primers used for laccase promoter cloning.

<table>
<thead>
<tr>
<th>Name</th>
<th>5′-3′ sequence</th>
<th>Orientation on promoter</th>
<th>Gateway recombination site</th>
<th>Promoter Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAC3 F1</td>
<td>aagtcaacttcacgatattacg</td>
<td>F</td>
<td>attB1</td>
<td>1.44 kb</td>
</tr>
<tr>
<td>LAC3 R1</td>
<td>tttgatcccttactgtgagaaag</td>
<td>R</td>
<td>attB2</td>
<td>1.44 kb</td>
</tr>
<tr>
<td>LAC4 F2</td>
<td>gtttgtcttactttgcttc</td>
<td>F</td>
<td>attB1</td>
<td>1.95 kb</td>
</tr>
<tr>
<td>LAC4 R1</td>
<td>gctacctctcttactttgcttc</td>
<td>R</td>
<td>attB2</td>
<td>1.95 kb</td>
</tr>
<tr>
<td>LAC5 F2</td>
<td>cagacatgtgtgagatagtggagaggag</td>
<td>F</td>
<td>attB1</td>
<td>1.9 kb</td>
</tr>
<tr>
<td>LAC5 R1</td>
<td>tctctctctcttttctcttc</td>
<td>R</td>
<td>attB2</td>
<td>1.9 kb</td>
</tr>
<tr>
<td>LAC12 F1</td>
<td>ggaatgttgcattctatgaagcttc</td>
<td>F</td>
<td>attB1</td>
<td>1.65 kb</td>
</tr>
<tr>
<td>LAC12 R1</td>
<td>ttgaagtttgaagcttttggag</td>
<td>R</td>
<td>attB2</td>
<td>1.65 kb</td>
</tr>
</tbody>
</table>

attB1: ggggacaagtttgtgacaaaaagcaggct
attB2: ggggacactttttgacaaagctttggag
### Table 5.11: Primers used in quantitative RT-PCR.

<table>
<thead>
<tr>
<th>Name</th>
<th>5’-3' sequence</th>
<th>Tm (°C) of the product</th>
</tr>
</thead>
<tbody>
<tr>
<td>3Fw-TT10</td>
<td>gtataaaaatacctaccgactgctcctagg</td>
<td>82.7</td>
</tr>
<tr>
<td>3Rev-TT10</td>
<td>cagaatcctgctattggtgcctcct</td>
<td>82.4</td>
</tr>
<tr>
<td>qEF F</td>
<td>cttgaggtttgagcgtggtat</td>
<td></td>
</tr>
<tr>
<td>qEF R</td>
<td>ccaagggtgaagaccaagaaga</td>
<td></td>
</tr>
<tr>
<td>APT1 F</td>
<td>cctataaggcgttgctattg</td>
<td>85.2</td>
</tr>
<tr>
<td>APT1 R</td>
<td>tcttcactcctactcctgtc</td>
<td></td>
</tr>
<tr>
<td>TUB4a F</td>
<td>ttcttcaggtttctttctttcc</td>
<td>83.2</td>
</tr>
<tr>
<td>TUB4a R</td>
<td>cctccattgctccaaacaccatac</td>
<td></td>
</tr>
</tbody>
</table>

### Table 5.12: Primers used for genotyping of SALK T-DNA mutants.

Primer pairs were designed with T-DNA Primer Design available at [http://signal.salk.edu/tdnaprimers.2.html](http://signal.salk.edu/tdnaprimers.2.html).

<table>
<thead>
<tr>
<th>Name of the mutant line</th>
<th>NASC</th>
<th>SALK</th>
</tr>
</thead>
<tbody>
<tr>
<td>At1g71450 LP</td>
<td>cAAAATGCAATATGCAAGC</td>
<td>N657434 SALK_136922C</td>
</tr>
<tr>
<td>At1g71450 RP</td>
<td>aggaaagttgagagcgtctc</td>
<td></td>
</tr>
<tr>
<td>At2g24430 LP</td>
<td>gtcgcgaagctacgcttgct</td>
<td>N660776 SALK_025040C</td>
</tr>
<tr>
<td>At2g24430 RP</td>
<td>tccgttcgataatcctcgc</td>
<td></td>
</tr>
<tr>
<td>At3g15500 LP</td>
<td>taaacgcagcgctgggtgag</td>
<td>N514331 (D)/(R) SALK_014331</td>
</tr>
<tr>
<td>At3g15500 RP</td>
<td>aaaaacaaacaaacactgg</td>
<td></td>
</tr>
<tr>
<td>At5g07700 LP</td>
<td>cttcgcagcttgtcgtcc</td>
<td>N662521 SALK_055242C</td>
</tr>
<tr>
<td>At5g07700 RP</td>
<td>atgtcagctgtacgcatcgac</td>
<td></td>
</tr>
<tr>
<td>At5g62320 LP</td>
<td>ggattaacaagctcgtcagtgg</td>
<td>N503193 SALK_003193 (F)</td>
</tr>
<tr>
<td>At5g62320 RP</td>
<td>tcaagtcaggtgagactttctaag</td>
<td></td>
</tr>
<tr>
<td>At5g66070 LP</td>
<td>catgcagctcactgctgtc</td>
<td>N658053 SALK_148182C</td>
</tr>
<tr>
<td>At5g66070 RP</td>
<td>ctaagtcagctcactgctgtc</td>
<td></td>
</tr>
<tr>
<td>Sig LB1</td>
<td>cggaaccacaccctcagactcag</td>
<td>nd nd</td>
</tr>
</tbody>
</table>

### Table 5.13: Other primers used in the study.

<table>
<thead>
<tr>
<th>Name</th>
<th>5’-3' sequence</th>
<th>Used for:</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF1 Alpha A4 UP</td>
<td>ATGCCCAAGGACATCCTGATTTTAT</td>
<td>expression control for RT-PCR</td>
</tr>
<tr>
<td>EF1 Alpha A4 LOW</td>
<td>TGGGCGGCTCGCTAGTGGATCA</td>
<td>constructs verification and sequencing in pBIB-Hyg vector</td>
</tr>
<tr>
<td>nos3A</td>
<td>CATCGCAAGACGGCCACAGGG</td>
<td>constructs verification and sequencing in pBIB-Hyg vector</td>
</tr>
<tr>
<td>pBIB Hyg</td>
<td>CCATTGAAGGCCCCCTGACAGCTACC</td>
<td>constructs verification and sequencing in pBIB-Hyg vector</td>
</tr>
<tr>
<td>UP</td>
<td>GTAAACGAGGGCCAGGT</td>
<td>Used for pTOPO and pBS construct verification and sequencing</td>
</tr>
<tr>
<td>RP</td>
<td>GGAAAAGCTATGGAGACCGT</td>
<td>Used for pTOPO and pBS construct verification and sequencing</td>
</tr>
<tr>
<td>GUS1</td>
<td>CTGATCAATTCCACAGTTTTCGCG</td>
<td>Used for GUS constructs verification and sequencing</td>
</tr>
</tbody>
</table>
### 5.2. PRIMERS

Table 5.14: CATMA GST primers used to study expression of flavonoid biosynthesis genes.

<table>
<thead>
<tr>
<th>Name</th>
<th>5’-3’ sequence</th>
<th>AGI</th>
<th>CATMA GST</th>
<th>PROBE / PCR product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT1 GST 5</td>
<td>TGGAGTCACCACCATATACGAGA</td>
<td>Atg34790</td>
<td>CATMA1a33100</td>
<td>158</td>
</tr>
<tr>
<td>TT1 GST 3</td>
<td>CAGTTTATCGGCAATATAGGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT2 GST 5</td>
<td>TTTCAAGTAAGTCACCACCATATACGAGA</td>
<td>Atg55550</td>
<td>CATMA5a30760</td>
<td>207</td>
</tr>
<tr>
<td>TT2 GST 3</td>
<td>CAACAGTAAGTCACCACCATATACGAGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT3 GST 5</td>
<td>GAACAGGAGCGCATATTC</td>
<td>Atg54280</td>
<td>CATMA5a38610</td>
<td>191</td>
</tr>
<tr>
<td>TT3 GST 3</td>
<td>TGGAGTTAGCTTATAATGCCTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT4 GST 5</td>
<td>TCTCGGGCTAGACCATCTTT</td>
<td>Atg51390</td>
<td>CATMA5a21150</td>
<td>400</td>
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<tr>
<td>TT4 GST 3</td>
<td>CGGAAAGAAGAAGAAGACGGAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT5 GST 5</td>
<td>GCCTGCTGAGAAAGAAGAGTAG</td>
<td>Atg35512</td>
<td>CATMA3a48130</td>
<td>221</td>
</tr>
<tr>
<td>TT5 GST 3</td>
<td>CAGGGTTCTTCTGGCTTATTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT6 GST 5</td>
<td>TTTGGACCAATGGGGAGGT</td>
<td>Atg351240</td>
<td>CATMA3a44230</td>
<td>271</td>
</tr>
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*Note*: The table lists the 5’-3’ sequences of the primers used in the study, along with their corresponding AGI numbers and the CATMA GST product sizes. The primers were used to study the expression of flavonoid biosynthesis genes.
5.3 Seeds colors

A. Ws-4 Ler Col-0 Cvi-0

B. tt10-2 tt10-3 tt10-1 tt10-4 tt10-5 tt10-6 tt10-7

Bay-0 Sha

Figure 5.2: Seed colors of various Arabidopsis accessions and tt10 mutant alleles, (A). Seed color of the plants used in the Ler ‘complementation’ experiment, (B).
Figure 5.3: Seed color of various mutants used in the study - Part 1.

Figure 5.4: Seed color of various mutants used in the study - Part 2.  
(A) Mutants chosen from co-expression studies.  
(B) Mutant carrying NahG gene and Ler wild-type control.

Figure 5.5: Seed color of mutants in laccase gene.
5.4 Map of the methylation in the *TT10* gene

Methylation map of the DNA in seedlings have been pictured by the Genome Browser at [http://epigenomics.mcdb.ucla.edu/](http://epigenomics.mcdb.ucla.edu/) (UCSC Genome Bioinformatics).

Figure 5.6: Methylation map of the *TT10* gene region.
5.5 Transposon in *TT10* promoter

Figure 5.7: Alignment of transposon sequence.

Alignment of the Col-0 wild-type intergenic region -2.2-kb to -1.0-kb of *TT10* promoter. In-del is a promoter fragment identified by Hirofumi Ishihara which is not present in *Ler*. Atcopia42 is a transposon sequence retrieved from FLAGdb++ found in *TT10* promoter region.


BIBLIOGRAPHY


