Transcriptional regulation of chloroplast ascorbate peroxidases in *Arabidopsis thaliana*

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List of Abbreviation

(E)GFP	(enhanced) green fluorescent protein
(Δ)Ct	(difference in) threshold cycles
×g	times gravitational acceleration,
A. thaliana	*Arabidopsis thaliana*
A.tumefaciens	*Agrobacterium tumefaciens*
ABI4	abscisic acid (ABA)-insensitive 4
ABRC	Arabidopsis Biological Resource Center
APX	ascorbate peroxidase
ARR2	type-A response regulator 2
AsA	ascorbate
At	*Arabidopsis thaliana*
BY-2	bright yellow
cDNA	complementary DNA
Col-0	Columbia-0
COP1	constitutively photomorphogenic 1
COR	cold regulated
CRY1/CRY2	cryptochrome 1/2
CTAB	cetrimonium bromide
Cu/Zn SOD	copper-zinc superoxide dismutase
Cyt b_6f	Cytochrome b_6f
Cyt *c*	cytochrome complex
DAB	3,3'-Diaminobenzidine
DBMIB	2,5-Dibromo-6-isopropyl-3-methyl-1,4-benzoquinone
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DET1	de-etiolated homolog 1
DHA	dehydroascorbate
DHAR	dehydroascorbate reductase
dNTP	deoxynucleotide
EX1/2	executor 1/2
Fd_{ox}/Fd_{red}	oxidized ferredoxin/ reduced ferredoxin
Fe/Fe(II)/Fe(III)	iron/ferrous iron/ferric iron
FeSOD	iron superoxide dismutase
flu	fluorescent, a mutant accumulating $^1O_2$ in chloroplast
FSD1, 2, 3	iron superoxide dismutase isoform 1, 2, 3
g	gram
GADPH	glyceraldehyde-3-phosphate dehydrogenase

GPX	glutathione peroxidase
GR	glutathione reductase
Grx	glutaredoxin
GSH/GSSH	reduced glutathione/oxidized GSSH
HY5	long hypocotyl 5
kb	kilo base pairs
Kcat	rate constant
KIN	cold induced
Km	enzymes affinitie
LB medium	lysogeny broth medium
LB/RB	Left/right border
LEC2	leafy cotyledon 2
LHCB	light-harvesting chlorophyll-a/b proteins
LHCII	light harvesting complex II
Lin	Lincomycin B
LTI	low temperature induced
m	micro
M	molar
MDA	monodehydroascorbate
MDHAR	monodehydroascorbate reductase
MEKK1	MEK kinase 1
min	minute
miR156/157	micro RNA 156/157
MKK1/2	mitogen-activated protein kinase kinase 1/2
MPK4/6	mapk phosphatases 4/6
mRNA	messenger RNA
MS medium	Murashige & Skoog medium
MV	N,N'-dimethyl-4,4'-bipyridinium dichloride
MYB	MYB proto-oncogene protein
MYC	c-Myc
n	nano
NADPH /$NADP^+$	reduced/oxidized nicotinamide adenine dinucleotide phosphate
NF	norflurazon
NTR	NADPH-dependent thioredoxin reductase
ºC	Celsius degree
OD600	absorbance at 600 nm
OsAPx1/2/…/8	APX isoform 1/2/…/8 in *Oryza sativa*
PCR	polymerase chain reaction
PHYA/B	phytochrome a/b
PIF	phytochrome binding factor
PQ	plastoquinone
pro	promoter
Prx	peroxiredoxin
PS I/II	photosystem I/II
PSB A/B	a gene encoding the D1

	protein of PS II
qRT-PCR	quantitative real-time PCR
RbohD/F	respiratory burst oxidase homolog D/F
RD	responsive to desiccation
RFU	relative fluorescence units
ROS	reactive oxygen species
s	second
sAPX	chloroplast stromal/mitochondria ascorbate peroxidase
SBP	squamosa-promoter binding protein
SE	standard error
Sm/SpR	Spectinomycin resistance
SOD	superoxide dismutase
SPL3/8	squamosa promoter binding protein-like 3/8
STN7	state transition 7, a kinase
TAE	Tris acetate EDTA
tAPX	chloroplast thylakoid ascorbate peroxidase
taq	*Thermus aquaticus*
TE	Tris EDTA
TL29	a chloroplastic homologue of ascorbate peroxidase with no ascorbate peroxidase activity
Tm	melting temperature
Trx	thioredoxin
U	Unit
UV light	ultraviolet light
WRKY	a transcription factor family, with WRKYGQK conserved motif
Y1H	yeast one hybrid
YEB medium	yeast extract broth medium
µs	microsecond

Amino acids and nucleic acids were abbreviated according to recommendations given by the IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN). The abbreviation and sequences of transcription factor binding motif are explained in the text.

Summary

In this project, the promoter regions of *sAPX* (At4g08390) and *tAPX* (At1g77490) were cloned to pHGWFS 7.0 vector and transform to *Arabidopsis thaliana*. The constructs allow the analysis of the promoter activities by measuring GFP and GUS. Three independent transformation lines were studied for each promoter in order to study the regulation by a spectrum of environmental stimulus.

The promoter of *tAPX* gene was found to be controlled by chloroplastic H_2O_2. Application of chemicals blocking photosynthestic electron transport upstream of photosystem I suppressed the *tAPX* promoter activity. However, methyl viologen causing chloroplast reactive oxygen species production increased the *tAPX* promoter driven reporter expression. mRNA levels of *tAPX* in *2cpa*, *2cpb*, and *2cpa2cpb* mutants, which have higher chloroplastic H_2O_2, were higher than in wild type plants. These results suggest a role of chloroplastic H_2O_2 in regulating *tAPX* gene.

tAPX promoter activity was also shown to be elevated mechanic wounding. Although wounding caused cytosolic H_2O_2 production, wounding induction of of *tAPX* promoter activity was not primarily mediated by H_2O_2, since wounding in old leaves increased H_2O_2 but did not change *tAPX* promoter activity. Other factors, such as cold, drought, very strong high light, jasmonic acid or salicylic acid application, and exogenous feeding of H_2O_2, *etc*. made plants accumulate H_2O_2 in the cytosol, but did not activate the *tAPX* promoter. Taken these results together, chloroplatic but not cytosolic H_2O_2 regulate *tAPX* promoter.

tAPX promoter was also shown to be regulated by light in a spectrum specific manner. Long term exposure to dark completely suppressed the activity of the *tAPX* expression. Exposure of etiolated plants to 8 h white light, blue light, and far red light, but not red light recovered the *tAPX* promoter activity. These results suggest that chloroplast H_2O_2 and photoreceptor mediated signals together regulate the *tAPX* promoter.

Promoter truncation assays localized the important regulating region of *tAPX* promoter to -1295 to -734 bp upstream of translation start site. *In silico* prediction suggested that ARR2, ARR10, LEC2, SPL3, and SPL8 are putative transcription factors binding and regulating *tAPX*. Except LEC2, whose mutation caused a lethal phenotype, plants with T-DNA insertion within these genes are analyzed. mRNA levels of *tAPX* in *spl3* and *spl8* were shown higher than in wild type plants. Therefore, *spl3* and *spl8* are likely transcription factors negatively regulating *tAPX*.

sAPX was found less intensively expressed in leaf mesophyll cells. Greater promoter activity was observed in leaf veins. The promoter activity of *sAPX* in mesophyll cells and vasculature cells are differently controlled. Promoter was shown up-regulated by light in mesophyll cells, while down-regulated in leaf vasculature.

Generally, stresses like cold, drought increases *sAPX* promoter activity. Block of photosynthetic electron transport suppresses *sAPX* promoter. The photosynthesis regulation of *sAPX* promoter was related to ascorbate biosynthesis, as ascorbate biosynthesis is regulated by the carbohydrate availability which is influence by photosynthesis. Promoter truncation assay localized the regulation region of *sAPX* to be -654 to -408 bp upstream of transcription start site. Further analysis is needed to identify corresponding transcription factors.

Zusammenfassung

In der vorliegenden Arbeit wurden die Promotorregionen der plastidären Ascorbatperoxidasen, *sAPX* (At4g08390) und *tAPX* (At1g77490), in den Vektor pHGWFS 7.0 kloniert und in *Arabidopsis thaliana* transformiert. Diese Konstrukte ermöglichen die Analyse der Promoteraktivität durch GFP- und GUS- Messungen. Für beide Promotoren wurden je drei unabhängige Transformationslinien auf die Regulation durch verschiedenste Umwelteinflüsse untersucht.

Es konnte gezeigt werden, dass der Promoter von *tAPX* durch plastidäres H_2O_2 kontrolliert wird. Die Zugabe von Chemikalien, welche den photosynthetischen Elektronentransport zum Photosystem I blockieren, reduzierte die *tAPX* Promoteraktivität. Methylviologen, das die Produktion reaktiver Sauerstoffspezies in Chloroplasten fördert, steigerte die *tAPX* promoterabhängige Reportergenaktivität. Die *tAPX*-mRNA-Spiegel waren in den 2-Cys Peroxiredoxin-Deletionsmutanten *2cpa*, *2cpb* und *2cpa/2cpb*, die größere Mengen H_2O_2 in den Chloroplasten aufweisen, im Vergleich zu Wildtyppflanzen erhöht. Diese Ergebnisse deuten darauf hin, dass der *tAPX* Promotor durch plastidäres H_2O_2 reguliert wird.

Weiterhin konnte gezeigt werden, dass die Aktivität des Promotors durch mechanische Verwundung angeregt werden kann. Obwohl Verwundung die cytosolische H_2O_2 Produktion induziert, ließ sich die gesteigerte *tAPX* Promotoraktivität nicht alleine auf H_2O_2 zurückführen, da in verwundeten älteren Blätter die *tAPX* Promotoraktivität nicht beeinflusst wurde. Weitere Faktoren, wie z.B. Kälte, Trockenheit, starkes Licht, Jasmonsäure- oder Salicylsäureapplikation, sowie die exogene Zugabe von H_2O_2, führen ebenfalls zur Akkumulation von H_2O_2 im Cytosol, aber nicht zur Aktivierung des *tAPX* Promoters. Daraus lässt sich ableiten, dass plastidäres, aber nicht cytosolisches H_2O_2 den *tAPX* Promoter reguliert.

Der *tAPX* Promoter reagiert zudem spezifisch auf verschiedene Wellenlängen des sichtbaren Lichts: Längerfristige Anpassung an Dunkelheit reprimierte die *tAPX* Expression vollständig. Durch anschließende 8-stündige Belichtung mit weißem, blauem oder dunkelrotem Licht, aber nicht mit hellrotem Licht konnte die Promotoraktivität wiederhergestellt werden. Diese Resultate legen nahe, dass plastidäres H_2O_2 und Photorezeptor-vermittelte Signale gemeinsam den *tAPX* Promoter regulieren.

Mit Hilfe unterschiedlich langer, einseitig verkürzter Promoterfragmente konnte der regulatorische Bereich des Promoters auf -1295 bp bis -734 bp oberhalb des Startcodons eingegrenzt werden. *In silico* Prognosen zeigten ARR2, ARR10, LEC2, SPL3 und SPL8 als

potenzielle Transkriptionsfaktoren auf, die an den *tAPX* Promotor binden und ihn regulieren. Außer für LEC2, dessen Mangelmutante einen lethalen Phänotyp aufweist, wurden T-DNA Insertionslinien für all diese Faktoren untersucht. Die tAPx-mRNA-Spiegel waren in *spl3* und *spl8* Linien gegenüber Wildtyppflanzen erhöht. Daraus lässt sich ableiten, dass *spl3* und *spl8* Transkriptionsfaktoren die *tAPX* Expression negativ regulieren.

In Blattmesophyllzellen konnte kaum Expression von Reportern von *sAPX* Promotorfusionen nachgewiesen werden. Entlang den Blattadern war die Expression stärker. Außerdem wurde gezeigt, dass der Promoter im Mesophyll durch Licht induziert wird, während seine Aktivität entlang der Leitgewebe durch Licht reprimiert wird.

Grundsätzlich lässt sich sagen, dass Stressoren wie Kälte oder Trockenheit die *sAPX* Promotoraktivität induzieren, während eine Blockade des photosynthetischen Elektronentransports supprimierend wirkt. Die photosynthetische Regulation des *sAPX* Promoters wurde mit der Ascorbatbiosynthese in Zusammenhang gebracht, die unter Einfluss der Photosynthese durch die Kohlenhydratverfügbarkeit bestimmt wird. Durch Analysen von Reportergenlinien mit verkürzten Promotersequenzen konnte die für die Regulation der *sAPX* notwendige Region auf -654 bp bis -408 bp vom Startcodon lokalisiert werden. Dies ermöglicht es in weiterführenden Analysen die beteiligten Transkriptionsfaktoren zu identifizieren.

1 Introduction

1.1 Reactive oxygen species produced in chloroplast

Using chloroplasts and their associated function, plants are capable to utilize light energy to fix carbon dioxide (CO_2) by a series of electron transferring redox reactions. Chloroplasts, however, are challenged by energy input, especially when it becomes excessive. The inability of fixing all energy and electrons makes oxygen (O_2) prone to be excited by accepting electrons from photosynthesis intermediates, to generate singlet oxygen species ($^1O_2$) or superoxide anion (O_2^-). These can then be transformed to hydrogen peroxide (H_2O_2) and hydroxyl radical (HO·) (Apel and Hirt, 2004). These chemically active products are called reactive oxygen species (ROS).

1.1.1 Production and toxicity of ROS in the chloroplast

Inside plant chloroplasts, the photosynthetic electron flow is the major source of ROS (Figure 1.1). Accumulation of ROS occurs especially when plants encounter unfavorable environments. Although some of the ROS mediate cellular signaling to regulate gene expression and cellular processes (Thannickal and Fanburg, 2000), the accumulation of ROS can be harmful for plant cell (Sharma et al., 2012).

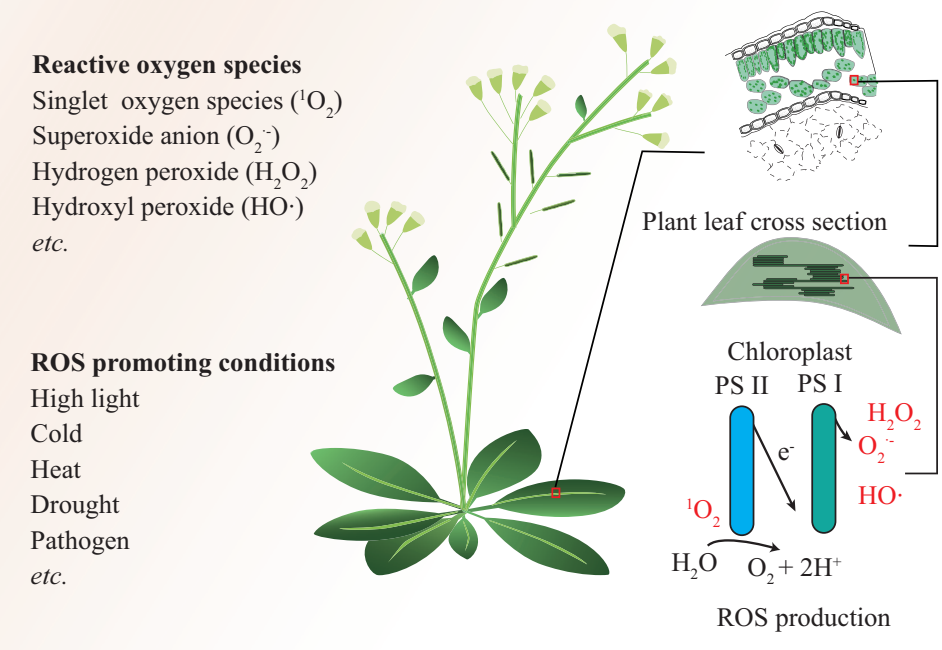

Figure 1.1 ROS generation in plant chloroplast. The major forms of ROS in plant cells are depicted. Stresses induce the accumulation of ROS. The ROS accumulation site in plant photosynthetic cells is shown in the right side of the picture. Red letters indicate ROS.

1.1.1.1 Singlet oxygen species ($^1O_2$)

$^1O_2$ is formed in the reaction centre of photosystem II and in the antenna system. Chlorophylls inside the light-harvesting complexes transform to the triplet state if there are not enough options to quench excess energy. Through a process of charge recombination reactions, triplet state chlorophylls transfer energy to molecular oxygen to generate $^1O_2$ (Krieger-Liszkay et al., 2008). This chemical has to be effectively scavenged; otherwise it damages the chloroplast or even the whole plant cell (Krieger-Liszkay et al., 2008). Cellular components, such as pigments, proteins, nucleic acids, and lipids are susceptible to $^1O_2$ (Halliwell and Gutteridge, 1985; Knox and Dodge, 1985; Triantaphylides et al., 2008). The thylakoid membranes, in which the photosystems are embedded, are an immediate target of $^1O_2$, because of their unsaturated fatty acid (Havaux, 1998). Proteins on the thylakoid membranes are vulnerable to $^1O_2$ oxidation. The oxidation and degradation of D1 protein by $^1O_2$ result in photoinhibition (Keren et al., 1997; Krieger-Liszkay, 2005). Signals triggered by $^1O_2$ may induce cell death (op den Camp et al., 2003). The un-scavenged $^1O_2$ may also transform to other form of ROS, such as H_2O_2 (Apel and Hirt, 2004).

1.1.1.2 Superoxide anion (O_2^-)

O_2 in chloroplasts is prone to be reduced to O_2^-, another form of ROS. The mid-point potential of O_2, which measures the electron taking ability, is lower than that of some photosynthetic intermediates. Thus O_2 is inclined to accept the electrons from photosystem I. The reaction of O_2 photoreduction was first discovered by Mehler and thus is called Mehler reaction (Mehler, 1951). O_2 accepts electrons from the electron donating site of photosystem I and generates O_2^-. O_2^- is also one of the major reasons of photoinhibition, which is characterized by light inducting decrease of photosynthetic capacity (Song et al., 2006).

1.1.1.3 Hydrogen peroxide (H_2O_2)

An enzymatic reaction catalyzed by superoxide dismutase (SOD) transforms O_2^- into H_2O_2. The production of this ROS in chloroplasts is in no way avoidable (Fridovich, 1978). Even at optimal condition, H_2O_2 is generated at the rate of 50 μM s^{-1} in chloroplast (Asada et al., 1977). The uncontrolled H_2O_2 oxidizes sulphur-containing amino acids, enzymes of the Calvin cycle and SOD (Kristensen et al., 2004; Groen et al., 2005). H_2O_2 is relatively stable compared to $^1O_2$ and O_2^-, with the half-life time of 1 ms (the half-life times for $^1O_2$ and O_2^- are 200 ns and 1 μs respectively) (Gorman and Rodgers, 1992; Reth, 2002). H_2O_2 is a mobile molecule readily transporting through membranes (Bienert et al., 2006). The mobility of H_2O_2

makes it a good signal molecule. It also makes it possible that it is detoxified at a cellular location distinct from its origin.

1.1.1.4 Hydroxyl radical (HO·)

Uncontrolled $O_2^{\cdot-}$ and H_2O_2 causes further destruction by generating HO·. According to Moller et al. (2007), HO· is more reactive than the above mentioned three ROS forms. The reaction is exacerbated if there are free metal ions, such as ferrous ion (Fe (II))(Haber and Weiss, 1932). Free metal ions are coming from degradation of metal binding proteins, such as proteins with Fe-S clusters. The reaction of H_2O_2 and Fe (II) (also called Haber-Weiss reaction) gives rise of HO·. The Fe (II) is reduced from Ferric ion [Fe (III)] by $O_2^{\cdot-}$ (Barb et al., 1951; Rush and Bielski, 1985). Thus, the free metal ion plays a catalyzing role in the net reaction (Equation 1.3). Other metals, such as manganese, and copper can react in the same way. The generation of HO· can be avoided by reducing the amounts of $O_2^{\cdot-}$, H_2O_2, and free metal ions.

$$\mathrm{Fe(II)} + \mathrm{H_2O_2} \rightarrow \mathrm{Fe(III)} + \mathrm{HO\cdot} + \mathrm{HO^-} \qquad \text{Equation 1.1}$$

$$\mathrm{Fe(III)} + \mathrm{O_2^{\cdot-}} \rightarrow \mathrm{Fe(II)} + \mathrm{O_2} \qquad \text{Equation 1.2}$$

$$\mathrm{H_2O_2} + \mathrm{O_2^{\cdot-}} \xrightarrow{\mathrm{Fe}} \mathrm{HO\cdot} + \mathrm{HO^-} + \mathrm{O_2} \qquad \text{Equation 1.3}$$

1.1.2 ROS production is promoted by environmental stress

Besides in the chloroplast, ROS are also produced in other plants compartments, such as mitochondria, peroxisome, cytosol, and the apoplast matrix (Tripathy and Oelmuller, 2012). In a functional cell, a normal metabolism produces ROS, and the production is enhanced in unfavorable conditions. ROS molecules are able to diffuse between compartments, which enables organelle communication (Bienert et al., 2006). However, unavoidable, the cellular production and accumulation of ROS vary with conditions (Figure 1.1). Generally, unfavorable conditions, such as excessive light, cold, water, nutrient limitation, or pathogen infection, increase plant ROS production by either increasing energy input, or decreasing the ability of processing it. The net output of the two antagonistic systems, ROS production and ROS scavenging, reflects the plant cell stress, and may be important to further control regulation.

Light intensity varies in different seasons within a year, in different hours within a day, in different minutes during a sun flecking hour, and thus provokes production of different amounts of ROS production according to the energy that reaches chloroplast (Tikkanen et al.,

2012). During excessive light exposure, over production of ROS causes photoinhibition (Tikkanen et al., 2012).

Upon exposure to low temperatures, plants lose their coordination between photoelectron flow and the Calvin cycle. As most of enzymes in the Calvin cycle are sensitive to decreasing temperature, electron fixing sink by Calvin cycle is reduced (Allen and Ort, 2001). The cold induced unfixed electron increases chloroplast ROS production (Wang et al., 2006b). ROS further target cellular structure (such as D1 protein, one of the PSII reaction center proteins), and cause photoinhibition of PS II (Wise, 1995). In chilling stress, photoinhibition caused by PS I, is seen (Zhang and Scheller, 2004).

In water deficient conditions, stomata close in order to reduce transpiration. Closing stomata limits CO_2 uptake. This slows down the Calvin cycle. Electrons beyond the sutured NADPH pool would go into ROS production (Scheibe et al., 2005).

ROS bursts are common in pathogen infected cells (Wojtaszek, 1997). The elevated ROS can attack pathogens, the host cells, or act as a signal to trigger the plant immune system (Torres et al., 2006). Activation of NADPH oxidases and peroxidases in the plasma membrane and the apoplast matrix lead to an oxidative burst and to defense against pathogens in the early stage of infection (Grant et al., 2000). Photorespiration is a series of reactions occurring in chloroplasts, peroxisomes, mitochondria, and cytosol. It is also a source of H_2O_2 in plant cell (Foyer et al., 2009). Research showed that ROS in peroxisome during photorespiratory is a perquisite for hypersensitive response, which is characterized by rapid cell death (Montillet et al., 2005).

1.2 Chloroplast ROS prevention and scavenge systems

To maintain the deleterious ROS under control, plants deploy a delicate ROS production preventing system and exquisite antioxidant system to scavenge ROS.

1.2.1 ROS preventing system

Multiple layers of sophisticated mechanisms minimize ROS production in plants: Habitat selection permits plants to avoid ROS promoting environment temporally and spatially. Skiophyte (plants that endure or thrive best at lowered light intensity), for example, “select” their niches in the shade through evolution. Morphologically, plants reduce the leaf angle and secret wax on the leaf surface to ward off excessive light. Excessive excitation can be further prevented by chloroplast movement. Even when the light eventually reaches the chloroplasts, large amounts of energy are fended off the from photosystem reaction centers by antenna

pigments (refer to chlorophylls and carotenoids which are arranged around photosystems like "antenna") and dissipated as heat, which is called nonphotochemical quenching (Holt et al., 2004). State transition is a reversible phosphorylation and redistribution of light harvest complex to photosystem I and photosystem II, facilitated by STN7 (Bellafiore et al., 2005). Changes in the antenna structure and size optimize the electron transport and minimize ROS production (Shikanai et al., 2002). Upon long-term exposure to excitation pressure, photosystem stoichiometry is adjusted by modulation the amounts of photosystem centre proteins (Pfannschmidt et al., 1999). Cyclic electron flow suppresses the production of reactive oxygen species even when energy enters photosystems (Chow and Hope, 1998; Golding et al., 2004).

1.2.2 Antioxidant system in chloroplast

Concomitant with prevention against ROS, plants deploy an antioxidant network to scavenge ROS once they are produced. It is rather a complex combination of plant antioxidant which is comprised of non-enzymatic and enzyme antioxidants, deliberately arranged in term of quantity and localizations. The coordinating and synergic work within system facilitates to maintain ROS equilibrium in optimal condition and most stress conditions.

1.2.2.1 Non-enzyme antixoidant

1.2.2.1.1 Carotenoids and tocopherols

Various forms of carotenoids and tocopherols are the major hydrophobic non-enzymatic antioxidants in chloroplasts. Carotenoids include non-oxygenated carotenoid and oxygenated carotenoid (xanthophylls, including lutein, violaxanthin, neoxanthin and zeaxanthin) (Havaux et al., 2007). They are embedded in thylakoid lipids or associated with antenna proteins (Havaux, 1998). They remove $^1O_2$ and HO· through two mechanisms: In the first, quenchers (carotenoids and tocopherols) draw energy from $^1O_2$ and become excited. The excited quenchers eventually dissipate energy as heat. Consequently, $^1O_2$ returns to its ground state. In this process, there is no quencher consumed. Due to the large number of conjugated double bounds, carotenoids are the most efficient $^1O_2$ quenchers working in this way. In contrast, the second mechanism involves the consumption of quenchers. Tocopherols mainly (carotenoids is also able to) "sacrificially" incorporate $^1O_2$, yielding tocopherol radicals (Ramel et al., 2012a; Ramel et al., 2012b). The resulting radicals are recycled back by the assistance of other antioxidants, such as ascorbate. The ability of plants to evade $^1O_2$ attack highlights the importance of carotenoids and tocopherols. In agreement with their function, research showed

that plants with less carotenoid are highly photosensitive (Ouchane et al., 1997). Dall'Osto et al. (2006) demonstrated that LHCII isolating from lutein-less (lutein is a major form of carotenoids) lut2 Arabidopsis mutant was more sensitive to photodamage than that from wild type plants. Overexpression of β-carotene hydroxylase protects plants against $^1O_2$ mainly by the lateral mechanism (Gotz et al., 2002; Johnson et al., 2007). Research also indicated that plants and algae inhibited in tocopherol biosynthesis are vulnerable to $^1O_2$ stress (Havaux et al., 2005; Kruk et al., 2005).

1.2.2.1.2 Ascorbate and glutathione

Large amounts of ascorbate and glutathione provide soluble antioxidant protection for chloroplasts. Their concentrations in chloroplast are several or tens of millimoles (Foyer et al., 1983; Law et al., 1983). Ascorbate and glutathione removes H_2O_2 and ·HO and other radicals, and converts the two antioxidants to their corresponding oxidized forms. Ascorbate and glutathione are quite distinct from antioxidants discussed above, in that their antioxidant reaction and recycling need specific enzymes. Integration and coordination of them are critical for recycling the oxidized form. The ROS detoxifying and renewal mechanisms are to be discussed in “enzymatic antioxidants” section.

The quantity of the two antioxidants in chloroplast is maintained by collaborative work of biosynthesis, catabolism, recycling and transport.

Ascorbate (L-ascorbate, natural occurring form) is a six-carbon sugar derivative. It is mainly synthesized in mitochondria from D-glucose and transported to various compartments. Ascorbate is synthesized via multiple biosynthetic pathways, including the d-glucosone, d-galacturonate, myo-inositol and d-mannose /l- galactose pathways (reviewed by Valpuesta and Botella (2004); Gallie (2013)). The later pathway is extremely important for plants, as shown by lethality of *vtc2 vtc5* (Vitamin C-2 and VTC-5 are two homologues encoding GDP-l-galactose phosphorylase) double mutants (Dowdle et al., 2007). The synthesized ascorbate in mitochondria diffuses to the cytosol and is transported to the chloroplasts by carrier facilitated diffusion while the transport between the thylakoid lumen and the stroma is simple diffusion following concentration and pH gradient (Horemans et al., 2000). Interestingly, there might be an ascorbate/dehydroascorbate exchange diffusion mechanism in the chloroplast envelop. This indicates that cytosolic antioxidant system can contribute to recycle oxidized ascorbate (Beck et al., 1983).

Glutathione is a tripeptide: γ-glutamyl-cysteinyl-glycine. It is synthesized in two ATP consuming conjugation reactions: glutamate-cysteine ligase catalyzed formation of γ-

glutamylcysteine, and following glutathione synthetase catalyzed ligation of glycine (Meister, 1995). The first reaction is rate limiting step and is localized in the chloroplast (Meyer and Fricker, 2002; Preuss et al., 2014), while the latter happens both in the chloroplasts and the cytosol. Glutathione synthesis in cytosol and other organelles depends on the transport of γ-glutamylcysteine (Noctor et al., 2002), and thus the synthesis is influenced by chloroplasts.

Ascorbate and glutathione are versatile components, undertaking many functions besides the antioxidant function. Ascorbate is a cofactor of the xanthophyll cycle involved non-photochemical quenching which (see section 1.2.1) prevents ROS production. The regeneration of α-tocopheryl radicals, a product of quenching singlet oxygen by of α-tocopheryl, requires ascorbate. Moreover, ascorbate can donate electron to photosystems (Mano et al., 2004). Some enzymes such as chloroplast ascorbate peroxidase are stabilized by ascorbate. Chloroplast ascorbate peroxidase is attacked by H_2O_2, and loses its function irreversibly in the absence of ascorbate (Yabuta et al., 2002). Glutathione provides a sulfur source for plants (Leustek and Saito, 1999). Moreover, the nucleophilic nature of the thiol group enables glutathione to bind heavy metals, and to protect the plant against heavy metal stress (Grill et al., 1987; Rauser, 1990; Clemens et al., 1999; Ha et al., 1999; Vatamaniuk et al., 1999). The heavy metal chelating role is also performed by the oligomer of glutathione, phytochelatin, which is synthesized by phytochelatin synthase (Vatamaniuk et al., 2000).

The pool sizes of both ascorbate and glutathione are important for plants. Not only the deprival of the either one of these molecules results in lethality (Cairns et al., 2006; Dowdle et al., 2007), but a moderate amount decrease causes increased sensitivity to stress (Kushnir et al., 1995; Muller-Moule et al., 2004). Although ascorbate and glutathione are multi-function molecules, their antioxidant function make it necessary for plants to keep large pool size of them. Evidence for this assumption is: the capacity of further increase of pool sizes upon oxidative stress (May and Leaver, 1993; Yabuta et al., 2007; Urzica et al., 2012), the compensational increase of ascorbate and glutathione when other antioxidant is depleted (Kanwischer et al., 2005; Queval et al., 2009), and the compensatory fluctuation between glutathione and ascorbate (Foyer and Noctor, 2011). The redox state is determined by the mid redox poise and ration of the reduced and oxidized form of redox buffering pair. The redox states of ascorbate and glutathione pool are also important regulating signals, regulating a series of processes including cell division and cell growth (Foyer and Noctor, 2011).

1.2.2.1.3 Phenolic antioxidant

Phenolics are a large family of chemicals consisting of at least one hydroxyl group bonded directly to an aromatic hydrocarbon group, such as lignin, tanin, anthocynin, flavonoids. By this definition, ascorbate and α-tocophenol are also phenolics. Some other phenolics are also able to reduce H_2O_2 in a similar way as ascorbate, catalyzed by a broader substrate specificity enzyme, for instance, guaiacol-type peroxidases (Sakihama et al., 2002). This provides important antioxidant protection in plant vacuoles and the apoplast. Evidence also pointed out that several flavonoids found in chloroplast envelope can protect chloroplast from $^1O_2$ (Agati et al., 2007; Agati et al., 2012). Lignin is phenol polymer important for plant defense, and water uptake. The cross link of its monomers is accompanied with H_2O_2 reduction (Lee et al., 2013).

1.2.2.2 Enzymatic antioxidant system

Plenty of parallel and stepwise enzymatic reactions interweave the intricate antioxidant system in chloroplasts to quench the high redox potential species generated by photosynthesis and other metabolism and recycle the non-enzyme antioxidants (Figure 1.2). The strong reductants generated from PSI are capable to convert molecular oxygen to O_2^- through Mehler reaction (Mehler, 1951). Subsequently, superoxide dismutase (SOD) catalyzes the O_2^- disproportionation, yielding H_2O_2 and O_2. Unlike that of peroxisome, chloroplast antioxidant enzyme system is the lack of catalase. The produced H_2O_2 can be detoxified by ascorbate dependent or ascorbate independent pathways (Figure 1.2). (1) Ascorbate-dependent pathway: By using ascorbate (AsA) as a specific electron donor, ascorbate peroxidase (APX) converts H_2O_2 to H_2O, and generates monodehydroascorbate (MDA). MDA can either be recycled to AsA by monodehydroascorbate reductase (MDHAR) accepting the electron from NAD(P)H (nicotinamide adenine dinucleotide phosphate), or spontaneously disproportionate to AsA and dehydroascorbate (DHA). Dehydroascorbate reductase (DHAR) catalyzes the reduction of DHA to AsA, using GSH as reductant. Next, the oxidized GSH (GSSH) goes back to its reduced state with enzymatic reduction by glutathione reductase (GR), with NAD(P)H as electron donor. (2) Ascorbate-independent pathway: Peroxiredoxins (Prx) or glutathione peroxidase (GPx) catalyze H_2O_2 detoxifying reaction. Prx and GPx are disulfide containing enzymes which function in electron transfer via the reversible oxidation of two vicinal protein-SH groups to a disulfide bridge (Holmgren, 1989; Goyer et al., 2002). The Prx and GPx take external electron from thioredoxin (Trx) or other electron donors. The oxidized Trx is reduced by NAD(P)H catalyzed by NADPH-dependent thioredoxin reductase (NTR) or reduced by ferredoxin (Fd_{red} and Fd_{ox} refer reduced and oxidized form respectively) by

ferredoxin-thioredoxin reductase (FTR) (Schurmann and Buchanan, 2008). Another H_2O_2 scavenging pathway uses reducing power from small redox proteins, such as glutaredoxin (Grx) or Trx, catalyzed by GPX which is followed by GR facilitating GSH recycling. This pathway is common in animal but not in plant, as GPX in plants does not contain selenium and only catalyze H_2O_2 detoxification at very low rate (Foyer and Noctor, 2000).

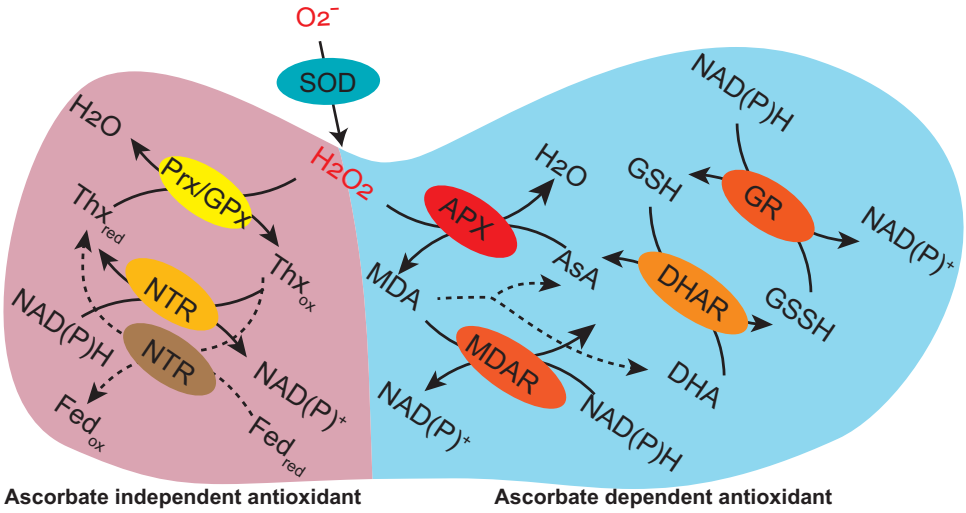

Figure 1.2 The chloroplastic enzymatic antioxidant system. SOD: superoxide dismutase, AsA: ascorbate; APX: ascorbate peroxidase; MDA: monodehydroascorbate; MDHAR: monodehydroascorbate reductase; NAD(P)H/NAD(P)+: reduced/oxidized nicotinamide adenine dinucleotide phosphate; GSH/GSSH: reduced glutathione/oxidized GSSH; DHA: dehydroascorbate; DHAR: dehydroascorbate reductase; Prx: peroxiredoxins; GPx: glutathione peroxidase; Trx: thioredoxin; NTR: thioredoxin reductase; Fdred/Fdox: reduced ferredoxin/oxidized ferredoxin; FTR: ferredoxin-thioredoxin reductase; Grx: glutaredoxin.

Although functionally redundant, the two pathways work simultaneously in chloroplasts. Dominant role of one pathway out of others might be seen because of enzyme abundance and efficiency. Ascorbate-glutathione pathway, as pointed out by Asada (1999), is of particular importance. For the chloroplast H_2O_2 scavenging enzymes (Table 1.1), the enzymes affinities (*Km* value) towards electron donors are well below the respective electron donors' availability. Moreover, the affinities towards H_2O_2 are comparable. Thus, the determination factor on to which pathway H_2O_2 flux is the rate constant (*Kcat*) and concentration of each enzyme. The *kcat* for ascorbate peroxidase is about 2 or 3 orders of magnitude larger than the two others (Table 1.1). Therefore, ascorbate-glutathione system is much more efficient, assuming local concentrations for the two pathways are the same. With agreement, measurement of enzyme activity demonstrated APX activity is as two folds as that of Trx based H_2O_2 removing enzymes (Dietz et al., 2006). However, gene expression data and gene

copy numbers in genome analysis pointed some low plant, such as *Selaginella moellendorffii* and in *Physcomitrella patens* favors Prx based pathway than ascorbate-glutathione pathway (Pitsch et al., 2010). Yet in one organism, pathway preference may also vary with environment cues. There seems to be an internal coordinating system partitioning the relative abundance of the two systems according to environment. Differential responses to stresses are seen in terms of transcript abundance (Mittler et al., 2004). For instance, upon cold stress, two FeSOD, FSD2 and FSD3 were down regulated, while one FeSOD, FSD1 in upregulated. This indicates that each pathway may have their specific role on certain stress. Compensatory rise of one pathway enzymes is seen when another is deficient (Baier et al., 2000; Kangasjärvi et al., 2008). The two pathways are associated and interconnected by sharing reducing power from GSH or NAD(P)H, whose pool sizes and redox states are recognized as important signals (Foyer and Noctor, 2011). The delicate arrangement of enzymes contributes to the efficiency of the overall system. Reports suggested that 10% and 30% of electrons from linear electron transport in algae and C3 plants goes to this system, so as to release the excessive energy (Biehler and Fock, 1996; Lovelock and Winter, 1996).

Table 1.1 Affinity and rate constant comparison of some chloroplast antioxidant enzymes

Enzyme	*Km* H_2O_2 (μM)	Km reductant (μM)	*Kcat* H_2O_2 (s^{-1})	*kcat/km* H_2O_2 (M^{-1} s^{-1})
Ascorbate-dependent pathway				
Tobacco stromal APX[a]	22±1	395±27	2510±41	$(1.1±0.1)×10^8$
Spinach APX[b]	30	300	290	$1.0×10^7$
Ascorbate-independent pathway				
Arabidopsis 2-Cys Prx A[c]	12±4	17±1	0.27	$(2.2±0.1)×10^4$
Pea 2-Cys PRX[d]	27.6	N.A.	0.69	$2.5×10^4$
Populus Prx IIE[e]	21.7±5.8	N.A.	0.57± 0.04	$(2.6±0.7)×10^4$
Barley 2-Cys Prx[f]	2.1	N.A.	0.23	$1.1×10^5$
Arabidopsis GPX1[g]	17.1±0.8	4.0 ± 0.4	0.83	$4.9×10^4$

NOTE a: Kitajima et al. (2006); b: Nakano and Asada (1987); c: König et al. (2013); d: Bernier-Villamor et al. (2004); e: Gama et al. (2008); f: Horling et al. (2003); g Iqbal et al. (2006). The concentration of ascorbate, thioredoxin, and glutathione are 10-50 mM (Foyer et al., 1983), 100-160 uM (Scheibe, 1981), and 5mM (Law et al., 1983) respectively.

Another layer of the complexity of the chloroplast enzymatic antioxidant system is reflected by presence of multiple isoforms of enzymes. Genes encoding chloroplast antioxidant enzymes underwent various extend of duplications in the course of evolution (Pitsch et al.,

2010). Subtle phenotype differences between mutants lack of different isoforms provided insight that the duplication of antioxidant enzymes not only brought partial redundancy but also subfunctionalization. The functional divergence may root from the sub-chloroplast localization of enzymes as well as the expression regulation. Function of antioxidant enzyme is affected by the micro-environment (pH, substrate availability, etc.) of its sub-chloroplast localization. A lumen localized isoform, APX4, has no peroxidase activity because of the highly acidified environment in the lumen (Granlund et al., 2009). Higher contribution of thylakoid isoform (tAPX) than stroma isoform (sAPX) in scavenging H_2O_2 was suggested by Asada (1999). Consistently, more severe damage was observed mutant in absence of the thylakoid isoform than that lack of the stroma isoform (Kangasjärvi et al., 2008; Maruta et al., 2010).

1.2.3 The APX family

APXs (EC 1.11.1.11) are peroxide scavenging enzymes specifically using ascorbate as the electron donor. To catalyze the conversion of peroxide to corresponding less toxic or non-toxic product, the enzyme is firstly oxidized to an intermediate, compound I. It is subsequently reduced to compound II and APX by the specific electron donor-ascorbate (Dunford and Stillman, 1976). Other peroxidases share the similar mechanism with APX and only different in substrates and electron donor specificity.

$$\mathrm{APX} + \mathrm{H_2O_2} \xrightarrow{k_1} \mathrm{Compound\ I} + \mathrm{H_2O} \qquad \text{Equation 1.4}$$

$$\mathrm{Compound\ I} + \mathrm{H_2S} \xrightarrow{k_2} \mathrm{Compound\ II} + \mathrm{HS^{\cdot}} \qquad \text{Equation 1.5}$$

$$\mathrm{Compound\ II} + \mathrm{H_2S} \xrightarrow{k_3} \mathrm{APX} + \mathrm{HS^{\cdot}} + \mathrm{H_2O} \qquad \text{Equation 1.6}$$

Note: S stands for reductant such as ascorbate. H_2S and $HS^{\cdot}$ stand for reduced and oxidized form respectively.

The high amount of ascorbate in the chloroplast and the inevitable production of hydrogen peroxide and its toxicity to photosynthetic enzyme in chloroplast led the speculation of the APX existence. Asada et al. (1977) estimated that the hydrogen peroxide production through superoxide-generating Mehler reaction is 50 μM s^{-1} (given that the chlorophyll concentration is 25 mM, and 10% of the photoreducing equivalents are used for hydrogen peroxide producing). If not scavenged, this amount of hydrogen peroxide is enormously damaging to the photosynthetic apparatus (Kaiser, 1979). Catalase is unlikely detoxifying hydrogen peroxide in the chloroplast, because of its nature of being inhibited by ascorbic acid (Orr, 1967a, b), whose concentration in chloroplast is as high as 50 mM (Gerhardt, 1964; Walker,

1971). Foyer and Halliwell (1976) proposed an ascorbate-glutathione-NADPH system trapping electrons from photosystem I (later known as water-water cycle), in which the hydrogen peroxide is reduced to water by using electrons from ascorbate. Although ascorbate can remove hydrogen peroxide itself, the necessity of an enzymatic conversion is not eliminated as the non-enzymatic speed is too low to have a biological relevance. Enzymatic studies soon confirmed that a lamellae bound enzyme has high ascorbate affinity normalizing hydrogen peroxide much more efficiently than the heat-stable non-enzymic peroxidative factor in plant extract (Groden and Beck, 1979).

After the first characterized APX from the chloroplast thylakoid (Groden and Beck, 1979), other APXs were found, such as cytosolic (Chen and Asada, 1989), mitochondrial (Jimenez et al., 1997), and peroxisomal APX (Yamaguchi et al., 1995; Bunkelmann and Trelease, 1996; Jimenez et al., 1997). APXs were also detected in various tissues, such as roots, the shoot apex, and germinating seeds (Asada, 1992; Gadea et al., 1999). The wide distribution of APXs in those compartments which could alternatively detoxify hydrogen peroxide by catalase or cytochrome peroxidase demonstrates the complexity of plant antioxidant system and the essentiality of APXs for plants.

1.2.4 The binding specificity, reaction mechanism, and inactivation of APX

APX are haem binding proteins and classified as Class I peroxidase based on the similar sequence to yeast cytochrome *c* peroxidase. Haem binds to APX through a co-ordinate bond to one histidine and a hydrogen bond to another histidine (Sharp et al., 2003). Ascorbate binds to a propionated side of haem and its adjacent basic residues of APX.

The reaction center of APX is comprised of the haem, haem binding sites, and related apoprotein. Sequence comparison between APX and yeast cytochrome *c* peroxidase revealed that two key basic residues (Arg172 and Lys30) are critical for the ascorbate binding specificity. The lack of corresponding acidic residue (Asp34, Glu35 and Asp37) in cyt *c* peroxidase explains why ascorbate does not bind to cyt *c*. As discussed in section 1.2.3, the compound I which contains an oxidized porphyrin by hydrogen peroxide is subsequently reduced by the electrons derived from ascorbate.

In the absence of ascorbate, oxidized haem in APX, especially chloroplastic APX, forms an irreversible cross-link to a distal tryptophan residue, leading to a repositioning of the haem. The cross-linked APX loses its ability to bind ascorbate, leads to the formation of radical sites inside the protein. This hydrogen peroxide mediated inactivation of APX is generally more severe in chloroplastic APX than in cytosolic APX. Compared with chloroplastic APX, the

loop structure in the vicinity of the propionate side chain of the porphyrin of cytosolic APX has an additional 16-residue insert. The bigger loop binds propionate chain noncovalently so as to donate electron more rapidly to the oxidized porphyrin than chloroplastic APX, for which reason the cross-link to tryptophan is slower (Kitajima et al., 2006; Kitajima, 2008). *In vitro* studies demonstrated both chloroplast ascorbate peroxidases are prone to be inactivated by plant oxidative stress (Miyake and Asada, 1996; Shikanai et al., 1998; Mano et al., 2001). The presence of ascorbate alleviates the inactivation (Hossain and Asada, 1984; Mano et al., 2001). Given that ascorbate is fairly abundant in the chloroplast, some researchers doubt whether the H_2O_2 medicated inactivation of chloroplast APX actually occurs *in vivo*. Liu et al. (2008) reported heavy metal inactivation of chloroplast ascorbate peroxidase. They pointed out that the inactivation of APX led to a loss of Rubisco (an enzyme involved in the first major step of carbon fixation in chloroplast) activity.

1.2.5 Physiological role of APX

Chloroplast ascorbate peroxidases are considered as key enzymes in the H_2O_2 scavenging system in chloroplasts (Asada, 1999). However, it is not surprising that the loss-of-function mutation of sAPX or tAPX does not give any obvious phenotype when the plant is grown under normal condition, now that chloroplasts are equipped with complex and redundant antioxidant network. Even the double mutant *sapx tapx* grows well under optimal condition (Kangasjärvi et al., 2008). The complimentary induction of other antioxidant genes eliminates risk of accumulation of ROS (Kangasjärvi et al., 2008). For instance, due to the diffusion property of the ROS, stromal ROS can be removed by the cytosolic isoform of APX. APX1 is sufficient to protect the chloroplast against oxidative stress under the experimental high light conditions (Davletova et al., 2005).

Research also pointed out that there may be other yet unkown unspecific ascorbate-dependent peroxidases other than sAPX and tAPX in chloroplasts (Giacomelli et al., 2007), when they observed that *sapx vtc2* (VTC2, ascorbate deficient) and *tapx vtc2* plants suffered more photodamage than *sapx tapx*. The protective role is seen under oxidative stress conditions such as high light, or ROS generating herbicides treatment. Mutant plants suffer more oxidative damage and show reduced photosynthesis when they grow under stress, as seen from accumulation of higher amount of ROS, oxidized protein and reduced photosynthetic rate (Kangasjärvi et al., 2008; Maruta et al., 2010; Maruta et al., 2012).

Accordingly, the overexpression of sAPX or tAPX enhances the tolerance to stresses (Murgia et al., 2004; Pang et al., 2011; Skirycz et al., 2011). Introduction of pea sAPX to cotton

protects the photosynthetic apparatus during chilling stress (Payton et al., 2001; Kornyeyev et al., 2003). Researchers agree that the loss function of sAPX and tAPX have different impact on plant. s*apx* was partly defected in its greening process when it was grown on methyl viologen supplemented medium, while *tapx* looked the same as wide type (Kangasjärvi et al., 2008). Maruta et al. (2010) claimed that tAPX is more important for protection against photooxidative stress in adult plants, as evidence was that higher level of accumulated of H_2O_2 and oxidized protein was in *tapx* than in *sapx*.

Apart from the ROS scavenging role of APX, it modulates the cellular signal that regulates gene expression. Alternation of gene expression, especially the expression of stress related genes was observed in APX knockout lines (Kangasjärvi et al., 2008; Maruta et al., 2010). RNAi induced transient knockout of tAPX had a distinct gene expression profile from stable T-DNA knockout lines (Maruta et al., 2012). The long-term acclimation of plants which involves reprogramming of gene expression help the plants to be more prepared for stress. $^1O_2$-mediated cell death and growth inhibition in *flu* mutant (Fluorescent, a mutant accumulating $^1O_2$ in chloroplast) was exacerbated in *flu* mutant overexpressing tAPX (Laloi et al., 2007), as also reflected by their gene expression profiles. This indicates that tAPX regulated H_2O_2 cross-talk with $^1O_2$-mediated signaling of stress responses. Similarly, H_2O_2 originated from chloroplast triggered a signal cross-talk with that triggered by cytosolic H_2O_2. The mutant deficient in both cytosolic APX1 and tAPX is distinct from the single mutants in several characteristics, such as flower time, protein oxidation during light stress, and accumulation of anthocyanins (Miller et al., 2007). Interestingly, *tapx* triggers signals that increase the plant resistance to heat stress. H_2O_2 produced in chloroplast is not able to induce the expression of some “ROS marker genes” (genes induced by ROS) as cytosolic H_2O_2 (Miller et al., 2007). Thus, the chloroplasts APX function as a specific gene expression regulator different from cytosolic ones.

1.2.6 Phylogeny of APX

Ascorbate peroxidases were classified as a Class I peroxidase, the other members within this family are cyt *c* (cytochrome complex) peroxidase and catalase peroxidase (Welinder, 1992). The cytochrome *c* peroxidase and ascorbate peroxidase are closely related, and derived from same gene, KatG (Zamocky, 2004). It is yet unclear why this gene split into two peroxidases using different electron donors.

There are 8 genes coding for APXs in the Arabidopsis genome. APX1, APX2 and APX6 are cytosolic isoforms; APX3, APX4 and APX5 are peroxisomal isoforms; stromal sAPX and

thylakoid tAPX are chloroplastic isoforms with sAPX which can be also localized in mitochondria (Chew et al., 2003). The central region of the APX contains a catalytic active site and a haem binding domain (Henrissat et al., 1990). Phylogenetic analyses indicated that chloroplastic and non-chloroplastic APXs represented two distinct groups (Teixeira et al., 2004). The chloroplastic isoforms have a chloroplastic sorting peptide at their N-terminal. Furthermore, the thylakoid isoform has a transmembrane helix at its C-terminus. The membrane anchored peroximal isoforms have also transmembrane helix in their C-terminals.

There is another protein, TL29, who shares high sequence similarity with the two chloroplast APXs found in chloroplast (Kieselbach et al., 2000). This protein was demonstrated to have antioxidant capacity (Wang et al., 2014), however, it was clearly shown that it is not an ascorbate peroxidase as it lacks both the active site and the haem binding domain (Granlund et al., 2009). In some "lower" organism only one copy, either a stromal isoform or a thylakoid isoform for *Chlamydomonas reinhardtii* and for *Physcomitrella patens* respectively, is encoded (Pitsch et al., 2010). In tobacco, spinach and pumpkin, the two isoforms are encoded by the same gene, the transcript of which is destined to stroma or thylakoid isoform controlled by alternative splicing. More than one gene for sAPX or tAPX is presented in some higher organism, such as *Oryza sativa*. The diversity comes from repeated lateral transfer of a common ancestor ascorbate peroxidase gene from chloroplast to nucleus (Pitsch et al., 2010).

1.3 Regulation of chloroplast antioxidant enzymes

1.3.1 The plastid retrograde signalling network

Because chloroplast ascorbate peroxidases are proteins whose genetic information is encoded in the nucleus, it is necessary to consider the now emerging picture of the plant retrograde signaling network in order to understand the regulation of chloroplast APX. Plant retrograde signaling refers to that the signal from organelles regulates nucleus gene expression, while the nucleus-to-organelles control is called anterograde signaling (Koussevitzky et al., 2007). The endosymbiotic origin and gradual transmission of plastid DNA to nucleus through evolution make the chloroplast proteins a "mosaic of origin" (Keeling, 2010). Its proteins are either encoded and expressed in the chloroplast itself or encoded in nucleus and expressed in nucleocytosolic system. The expression of plastid genes are partly controlled by the chloroplast status and mostly by global regulation of transcription and translation activities (reviewed by Mayfield et al. (1995)). The regulation of plastid genes by nucleocytosolic system is called anterograde regulation. The activity and specificity of plastid gene expression

are also regulated by nucleocytosolic production of plastid nucleus encoded polymerase, plastid ribosomal proteins, and sigma factors. More than 95% of chloroplast proteins are encoded in the nucleus (Shi and Theg, 2013). The demand of those proteins is coordinating to the chloroplast status. Thus, signals originated in the chloroplast are translocated from the chloroplasts and regulate the gene expression in nucleus and cytosol.

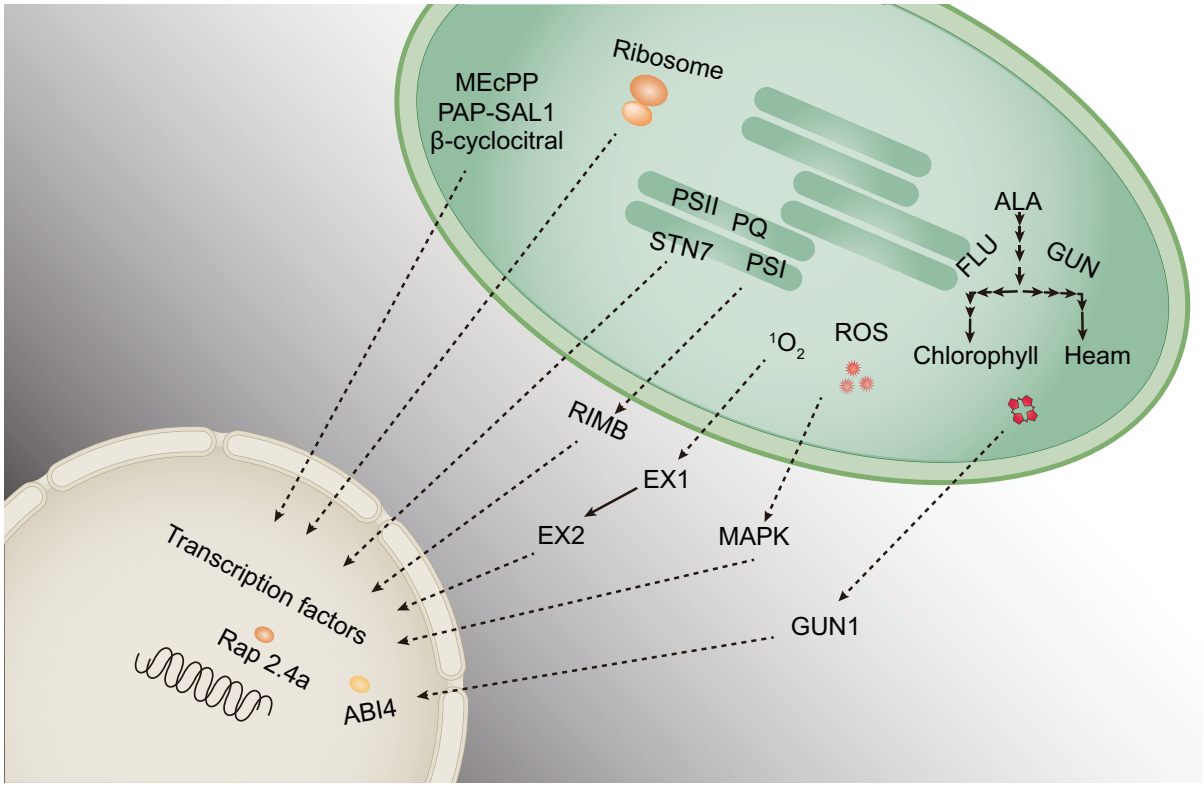

Figure 1.3 Retrograde signaling pathways. The metabolites in the chlorophyll or haem biosynthesis pathway produce a signal that activates a transcription factor ABI4 via GUN1. ROS production in the chloroplast activate a cascade of MAPK kinases, relays the signal to nucleus. Other mediators identified are EX1 and EX2 (EX2 is the downstream of EX1). Redox status of the electron transport chain, either in the PQ pool or PSII downstream acceptors generate signal for nucleus gene expression. STN7 is important for PQ pool redox state signal while RIMB are important for PSII downstream signals. The corresponding transcription factor of PSII downstream acceptor redox- RIMB signal is Rap2.4 a. The translation of protein in the chloroplast generate a signal called plastid gene expression signal, the downstream player is thought to be GUN1. Recently identified signal are MEcPP, PAP-SAL1, and β- cyclocitral.

Since the first indication of the existence of chloroplast-nucleus retrograde signaling in 1979 (Bradbeer et al., 1979), several distinct pathways that relay the chloroplast information to the nucleus have been revealed.

1.3.1.1 Tetrapyrrole pathway

The screen for mutants altered in expression of CAB and RBCS pattern led the discovery of *gun* (*genomes uncoupled*) mutant (Susek et al., 1993). Except for GUN1, the other GUNs are enzymes working in the tetrapyrroles biosynthesis pathway, which is important for chlorophyll, heam, siroheam, and phytochromobilin synthesis. Later researches suggested that it is the tetrapyrrole intermediate, Mg-protoporphyrin IX, regulating nucleus genes (Strand et al., 2003). However, how this metabolite signal transmitted is not yet known. One downstream factor of the GUN pathway has been identified as ABI4 (Abscisic Acid-Insensitive 4), an AP2 (Apetala 2) transcription factor (Koussevitzky et al., 2007). Interestingly, ABI4 regulates photosynthetic genes whose promoters contain a G-box motif. GUN1 is a pentatricopeptide repeat (PPR) protein, which is not associated with the tetrapyrrole pathway. GUN1 is suggested as a player in a signal transduction induced by impaired expression of plastid genes (Armbruster et al., 2010).

1.3.1.2 Plastid gene expression pathway

Impaired plastid genes expression generates another different signal which is known as PGE pathway (Plastid Gene Expression). Plastid gene expression can be specifically impaired in plants treated with chloramphenicol, lincomycin, or erythromycin. A signal generated through changes in the expression of nucleus encoded chloroplast genes (Rapp and Mullet, 1991; Sullivan and Gray, 1999). Researchers using mutants impaired in translation in chloroplast and mitochondria showed that the retrograde signal can only be produced when both the chloroplast and mitochondria are inhibited in translation (Pesaresi et al., 2006). Koussevitzky et al. (2007) suggested GUN1 lies downstream of PEG signals transduction and conjugating tetrapyrrole signal pathway. A recent study suggested that several NAC transcription factors may be the downstream mediators of PEG pathway (Gläßer et al., 2014). Several motifs shared by genes which are regulated by PEG signals have been suggested, including a G-box variants (Leister et al., 2014).

1.3.1.3 Redox signal from plastoquinone pool

Plastid redox state signal is another type of retrograde signals regulating nucleus encoded chloroplast genes. Redox state is the ratio of the inter-convertible oxidized and reduced form of a specific redox couple. Various redox state sources including intersystem electron transport in the chloroplast, particularly the plastoquinone pool (PQ), and the PSI acceptor site with a variety of redox-active components such as NADPH, thioredoxin, glutathione and

glutaredoxin are suggested as sources for retrograde signaling (Pesaresi et al., 2007). Some metabolite pairs and thiol/disulfide proteins were also proposed (Foyer and Noctor, 2009). The redox state reflects the oxidizing tendency in chloroplast. High light as well as other stresses can change the chloroplast redox state swiftly. Now, the best known player for redox state signal pathway is STN7. STN7 is a chloroplast kinase phosphorylating LHCII and involved in state transitions (section 1.2.1) and photosynthetic acclimation. It is activated by the reduced state of PQ pool and transmits a signal regulating chloroplast encoded nucleus gens (Pesaresi et al., 2009).

1.3.1.4 Redox state of acceptor availability of photosystem I related signal

Another type of players sensing or transduction redox state of acceptor availability of photosystem I, RIMB (Redox imbalance) are under investigation (Heiber et al., 2007). The screen of *rimb* mutants was based on altered expression of 2-Cys-peroxiredoxin A, whose expression was demonstrated as being regulated by redox state down stream of PSI (Baier et al., 2004).

1.3.1.5 Plastid-encoded RNA polymerase related signal

Another redox sensor lies in plastid-encoded RNA polymerase (PEP). PLASTID REDOX INSENSITIVE 2 is a protein associated with PEP. The mutation in this gene, as well as other genes involved in PEP, changes the expression of nucleus encoded proteins such as LHCB (Kindgren et al., 2012). Transcription factors binding gene promoter and regulating gene transcription in nucleus in a redox dependent manner are discovered (Shaikhali et al., 2008; Shaikhali et al., 2012).

1.3.1.6 Chloroplast originated reactive oxygen species related signal

The various forms of ROS ($^1O_2$, O_2^-, H_2O_2) produced in chloroplasts are demonstrated not only as deleterious chemicals but also as signal molecules. $^1O_2$ burst in *flu* mutant is under a dark-to-light shift switch. $^1O_2$ produces signals for regulating the expression of nucleus genes, of which many are cell death related (op den Camp et al., 2003). H_2O_2 production in chloroplasts generates a different signal that can antagonize $^1O_2$ triggered signal (Laloi et al., 2007). Interestingly, the expression of cytosolic APX2 as well as other genes are activated by the chloroplast generated H_2O_2 (Galvez-Valdivieso et al., 2009). EXECUTER 1 and EXECUTER 2 were shown to mediate $^1O_2$ triggered signal to the nucleus based on screening mutants in *flu* background (Wagner et al., 2004; Lee et al., 2007b). Another downstream

mediator, PLEIOTROPIC RESPONSE LOCUS 1, was revealed (Baruah et al., 2009). Mitogen-activated protein kinase (MAPK) is involved in transduction of signals generated from chloroplastic H_2O_2 (Liu et al., 2007).

1.3.1.7 Other signals and perspective

Although the chloroplast-nucleus retrograde signaling pathways are increasingly complex, intensive researches are being conducted in this field. New pathways such as 3'-phosphoadenosine 5'-phosphate (PAP), β-cyclocitral, methylerythritol cyclodiphosphate (MEcPP) have been revealed recently (Estavillo et al., 2011; Ramel et al., 2012a; Xiao et al., 2012). However, we do not have solid answers to the following questions now: (1) Are there more resources that trigger a retrograde signal? (2) How the signal is transmitted? (3) What is the corresponding target of a specific signal? (4) How does a retrograde signal cross-talk with other class signal transduction such as Ca^{2+} or phytohormones?

1.3.2 The regulation of chloroplast antioxidant enzymes

All the chloroplast antioxidant enzymes are encoded in the nucleus. The arrangement of the information and function site enables or requires combinational work or anterograde and retrograde signaling. Studies on both transcriptional and posttranscriptional regulation for each player of the chloroplast antioxidant network are now increasing. Posttranscriptional regulation for chloroplastic antioxidant enzymes, for instance, mRNA stability, translation rate, protein transport to the chloroplasts, incorporation with cofactors, is under investigation. The transcriptional regulation lies in the first few steps. Together with posttranscriptional regulation, the transcription regulation modifies the expression level of antioxidants genes to optimize the efficiency of chloroplast ROS scavenging system without wasting energy. Insight shed from microarray studies indicated the transcriptional regulation of each individual is not uniform (Mittler et al., 2004). Several genes coding for antioxidant enzymes are down regulated while others are up-regulated upon the same stress stimulus (Mittler et al., 2004). The transcription of these antioxidants is under control of different signal pathways. Transcriptional regulation study of each gene would possibly find out new signal pathways. For example, the transcriptional regulation study of the 2CPA gene identified a novel source of retrograde signal. The transcription factor would be the downstream target of the signal pathways. Recently, a transcription factor that negatively regulates sAPX transcription in response to the cellular redox state was discovered (Klein et al., 2012). The knowledge of the transcriptional regulation helps to understand how the antioxidant works as a whole system.

The two chloroplastic isoforms of APXs work at the downstream of SOD. The reaction starts a H_2O_2 removing cycle with MDAR and DHAR. The removing cycle is parallel with PRX and GPX mediated H_2O_2 removing cycles. Ascorbate and H_2O_2 are the substrates and products connecting this interlocked system. The amount of ascorbate, H_2O_2, and the redox state of ascorbate are potential regulator of chloroplast signals. A transcriptional regulation study would reveal how the enzymes are regulated coordinately.

1.3.3 Regulating role of phytahormones on antioxidant system

Evidently, there are regulation links between phytohormones and the plant antioxidant system. Firstly, application of certain phytohormones, such as IAA (indole acetic acid), ABA (abscisic acid), JA (jasmonic acid), and SA (salicylic acid), provokes ROS accumulation (Pei et al., 2000a; Pei et al., 2000b; Joo et al., 2001; Park et al., 2003; Hung et al., 2006; Kalachova et al., 2013). And the ROS produced in turn are crucial for the phytohormone signal transduction (Kwak et al., 2003; Hung et al., 2006; Kalachova et al., 2013). For example, the comparison of transcriptome changes of Arabidopsis response to ABA and H_2O_2 revealed the two stimuli shared one-third of regulon (Wang et al., 2006a). The disruption of H_2O_2 producing enzymes (AtrbohD and AtrbohF) impairs ABA signaled stomatal closure (Kwak et al., 2003). Besides regulating gene expression, ROS are also direct downstream players of phytohormone function, such as ROS function in auxin caused gravitropism (Joo et al., 2001). However, a transient rather than stable ROS accumulation is triggered by phytohormones, which means that a regulated antioxidant system works at the same time to ensure the ROS accumulation pulse is enough for signaling but not enough for causing cell damage and apoptosis. Secondly, certain phytohormones, such as SA, can directly target on the antioxidant enzymes thus modify its antioxidant capacity (Durner and Klessig, 1995). SA inhibits ascorbate peroxidase activities. In the coordinated system, the deactivation of one enzymes may coordinately affect the capacity of the whole system. Subsequently, adjustment of the abundance of other antioxidant enzymes is needed. On the other hand, the phytohormones can be induced by the ROS level of the plant cell (Tamaoki, 2008). Through sensing the ROS level, plants synthesize phytohormones coordinately and regulate the long term response to the environmental cues.

Not only the production but also the scavenging of ROS is under the control of phytohormone. The regulation of chloroplast 2CPA, for instance, was shown to be achieved on the platform provided by ABA signaling cascade (Baier et al., 2004). It is under debate whether it is primary or secondary effect of ABA on regulating nucleus encoded chloroplast

genes (Baier and Dietz, 2005; Gläßer et al., 2014). Transcriptomic and proteomic studies also provided insight that the chloroplast antioxidant system are co-regulated by phytohormones and chloroplast signals, such redox of PET (photosynthetic electron transport), ROS signal, and sugar, *etc.* (Sasaki-Sekimoto et al., 2006; Xing and Xue, 2012; Gläßer et al., 2014). The identification of ABI4 as a conjugating point for ABA signal and chloroplast retrograde signal encourages further investigation on the cross-talk of chloroplast retrograde signal and other phytohormone signal. ABI4, as an important ABA signal mediator, is located downstream of the nucleus encoded chloroplast genes master switch GUN1. It must be regulated of the antioxidant system to attenuate the ROS accumulation, so that plants utilize ROS signaling function and avoid ROS damage. The phytohormone regulation of chloroplast ascorbate peroxidases is of particular interest. For one thing, the APXs are of particular importance for the overall system (Polle, 2001). Moreover, the direct targeting and inhibition of APX by SA pointed that the APX might be the important mediator of the plant immune response caused by SA (Durner and Klessig, 1995). The cytosolic ascorbate peroxidase is also shown regulated by oxidative stress and ABA (Galvez-Valdivieso et al., 2009).

1.4 Aim of the present study

The present work aims to understand the transcriptional regulation of chloroplast ascorbate peroxidases (*sAPX*, at4g08390; and *tAPX*, at1g77490) in *Arabidopsis thaliana*. Using the reporter gene fused with *sAPX* and *tAPX* promoter, the promoter activity of the two genes in responses to stress cues is determined. A variety of abiotic factors, especially factors inducing ROS production and affecting chloroplast function are tested in order to identify the regulating signal source. This study also examines the putative transcription factor binding region and putative transcription factors. The results will provide new information on expression regulation of the chloroplast antioxidant network.

2 Material and Methods

2.1 Plant materials

Arabidopsis thaliana natural accessions Columbia-0 and its gene modified lines were used in this study. The gene modified lines here refers to promoter driven reporter gene lines, T-DNA (transfer DNA) knock out/down lines, and truncated promoter driven reporter gene lines. The Columbia-0 seeds were originated from Nottingham Arabidopsis Stock Centre. Section 2.3 shows how the gene modified plant materials were obtained.

2.2 Plant growth conditions

2.2.1 Sterile culture of Arabidopsis thaliana seedlings

Sterilization of Arabidopsis seeds were performed in sterile 1.5 mL microcentrifuge tubes. Seeds (less than 200 µL) were soaked in 70% (v/v) ethanol for 1 min and then in 25% (v/v) household bleach (Glorix, Lever Farbergé, The Netherlands) for 8 min. The seeds were afterwards rinsed with sterile water for five times.

Seeds were sown on sterile Murashige & Skoog medium (MS; Duchefa, Haarlam, The Netherlands) medium, pH 5.7, supplemented with 0.5% (w/v) phytagel (Sigma, Steinheim, Germany) and 0.5% (w/v) sucrose. Seeds were stratification at 4 ºC for 2 days in the dark before they were transferred to growth conditions.

The light and temperature environment for MS sown plants growth were provided by in a climate-controlled chamber (CU-41L4X; Percival Scientific Inc., United States). Unless otherwise indicated, the light and temperature regime was 10 h 120 µmol photons m^{-2} s^{-1} white light (from 9 am), 22 °C/14 h dark, and 18 °C.

2.2.2 Growth of mature Arabidopsis thaliana plants on soil

Seeds were sown on moisture soil (composed of 42.4% (v/v) P-soil (Einheitserde, Sinntal-Altengronau, Germany), 42.4% (v/v) T-soil (Einheitserde, Sinntal-Altengronau, Germany) and 15.2% (v/v) perlite (Perligran G; Knauf Perlite, Dortmund, Germany)) and stratification at 4 ºC for 2 days. After stratification, the pots were put in a growth chamber under 10 h 120 µmol photons m^{-2} s^{-1} white light (from 9 am), 22 °C/14 h dark, 18 °C for germination. Watering was conducted so as to keep the soil moist. After the seedlings were ab. 1 week old, each individual was transferred to one pot (9 cm in diameter). The plants were then again put to

growth chamber under normal growth condition. Irrigation was conducted in every two days to keep the soil moist.

If the plants were used for seeds germination, 5 weeks old were transfer to green house. Long light condition was applied. 14 h 120 µmol photons m^{-2} s^{-1} white light (from 9 am) / 14 h dark. Temperature was controlled as ab. 20 ºC. Watering was kept in every two days to keep the soil moist.

2.3 Gene modified plant materials

2.3.1 Obtained *pro*sAPX:EGFP-GUS and *pro*tAPX:EGFP-GUS plant constructs

T-DNA lines expressing eGFP (enhanced green florescence protein) and GUS (β-glucuronidase) under the control of *sAPX* promoter (1947 bp in length, sequence see supplementary Data 1) and *tAPX* promoter (1519 bp in length, sequence see supplementary Data 1) were taken from the group collection. Hereafter, they are named as *pro*sAPX:EGFP-GUS and *pro*tAPX:EGFP-GUS, respectively.

2.3.2 Generation of truncated promoter-reporter lines

To localize the promoter region critical transcriptional regulation, different factions of *sAPX* and *tAPX* promoter was cloned, constructed in a plasmid in a way that the expression of reporter gene can be driven by the promoter factions. The constructs were transformed to Arabidopsis via *A.tumefaciens* carried vectors. Hygromycin B resistance test was used to select the transformed plants. Through a series of propagation and selection, the homozygous lines that carry the promoter fraction-reporter genes were obtained.

2.3.2.1 Isolation of genomic DNA

Genomic DNA was extracted from 4 weeks old Arabidopsis rosette leaves using the CTAB (cetrimonium bromide) method (Stewart and Via, 1993). The purity of DNA was acceptable as indicated by that the A260/A280 was greater 1.8 (measured by NanoPhotometer P300). DNA concentration was diluted to the concentration of ab. 50 ng/µL.

2.3.2.2 PCR amplification of truncated promoter sequences

In order to clone the desired promoter sequences (Table 2.4), several pairs of primers were designed (Table 2.4).

Table 2.1 The primers used for amplification of promoter fragments

Gene	Name of the primer pair	Forward primer Reverse primer	Annealing temperature [°C]	Position in the promoter of gene	Expected amplificon length
tAPX	tAPx-741	TGGCACTTACCCAAGAGGAT	58.04	-741 to -1 bp	741 bp
		TGTCACGTACGGTGGCG	60.10		
	tAPx-528	TTGGCCCCCAAGAAAGC	60.00	-528 to -1 bp	528 bp
		TGTCACGTACGGTGGCG	60.10		
	tAPx-211	CCAATATACTCGGCCCATAAAC	58.26	-211 to -1 bp	211 bp
		TGTCACGTACGGTGGCG	60.10		
sAPX	sAPx-1219	GCCACTGGGATTCGAGTAA	57.09	-1258 to -40 bp	1219 bp
		GGAAGAGATACAGCCACGTCA	58.74		
	sAPx-654	AACCCGTCACCATTACCATC	58.01	-693 to -40 bp	654 bp
		GGAAGAGATACAGCCACGTCA	58.74		
	sAPx-378	CCTCAATAAGCCCAAGTGGA	58.57	-417 to -40 bp	378 bp
		GGAAGAGATACAGCCACGTCA	58.74		
	sAPx-222	ATGGACTTTATTGGTGCAACTCT	57.70	-261 to -40 bp	222 bp
		GGAAGAGATACAGCCACGTCA	58.74		

Using the chemicals and procedure in Table 2.5 and Table 2.6, different fragments of the *sAPX* and *tAPX* promoters were amplified with thermocycler (PEQLAB 96 universal gradient, Germany). The PCR product wad mixed with 4 µL 6 × loading buffer (650 mM Tris-HCl, pH 6.8; 50% (v/v) glycerol; 0.025% (w/v) bromophenol blue). After a brief shaking, the mix was loaded to agarose gel (1.2% agarose (w/v) in TAE buffer (Tris acetate EDTA buffer: 0.8 mM Tris-acetate pH 7.5, and 0.02 mM EDTA), heated to boil for jelling, supplemented with 0.5 µg mL ethidium bromide when the temperature drops to ab. 60 ºC, poured to a gel chamber fixed with hole-guiding combs), immersed in electrophoresis chamber with TAE buffer. The electrophoresis chamber was then supplied with constant voltage (110 V, Bio-rad PowerPac Basic Power Supply) for 30 min. The gel was subsequently detected for fluorescence under UV-light (Intas Gel iX Imager, Germany). The gel contains the desired sized band was sliced and stored in a microcentrifuge tube for cleaning up.

Table 2.2 Component of PCR reaction

Substance	Volume (µl/well)
10× Buffer[a]	2.00
5 mM dNTP[b]	0.5
10 µM Forward primer	0.5
10 µM Forward primer	0.5
Taq polymerase	1.0
Water	13.5
DNA Template	2.00
Final Volume	20.00

Table 2.3 PCR amplification steps

Steps		Temperature	Time
Pre-denaturartion		95 ºC	10 min
40 cycles	Denaturation	95 °C	15 sec
	Annealing	T[c] °C	30 sec
	Extension	72 °C	X[d] sec
Final Extension		95 °C	10 sec
Store		4 °C	--

Note:

a: 10× Buffer is composed of 160 mM ammonium sulfate, 1 M Tris-HCl, pH 8.3, and 0.10% Tween-20 (v/v); b: The mixture is composed of 5 mM dATP, 5mM dCTP, 5 mM dGTP and 5 mM dTTP; c: The temperature is normally 5 ºC below the Tm calculated for the primer pairs or else it was optimized by gradient PCR. d: The extension time is dependent on the expected length of amplicon, using 1 min for 1kb.

2.3.2.3 Clean up and recover the desired sequence from the gel

The gel containing DNA fragments of the *sAPX* and *tAPX* promoter sequences was recovered and cleaned with Invisorb® DNA clean up kit (STRATEC Biomedical AG, Germany), according to the protocol provided with the kit.

2.3.2.4 pCR8/TOPO enter cloning

The cleaned DNA was further TA cloned into pCR® 8/GW/TOPO® vector according to the instruction from its provider (Life technologies™). The resulting product of this cloning was transformed to TOPO 10 competent *Escherichia coli* cells using the heat shock method according to the manufactory's instruction. The cell was spread onto Petri dishes containing LB medium (Lysogeny Broth medium, 1% Tryptone (w/v), 0.5% Yeast extract (w/v), 1% NaCl (w/v), 1% agar(w/v)) supplemented with 100 mg/L spectinomycin. The Sm/SpR gene enables the transformed cell to survive and grow colonies on the plate. Medium inoculated with these cell was put in a 37 ºC incubator upside down for overnight cultivation. The colonies come out the next day were further confirmed their possession of pCR® 8/GW/TOPO® vector carrying promoter sequences in desired direction by colony PCR with M13 forward sequencing primer (-20): (GTAAAACGACGGCCAGT) and another reverse primer corresponding to the promoter sequences. The colony PCR is similar with PCR procedure described before except only that a small amount of colony plaque was used as template. The confirmed colonies were further inoculated into a 20 mL sterile vial contain LB

medium supplemented with 100 mg L^{-1} spectinomycin. It was put into 37 ºC shaking bed with 200 rpm for overnight cultivation. The overnight culture with an OD600 over 0.8 was ready for plasmid isolation.

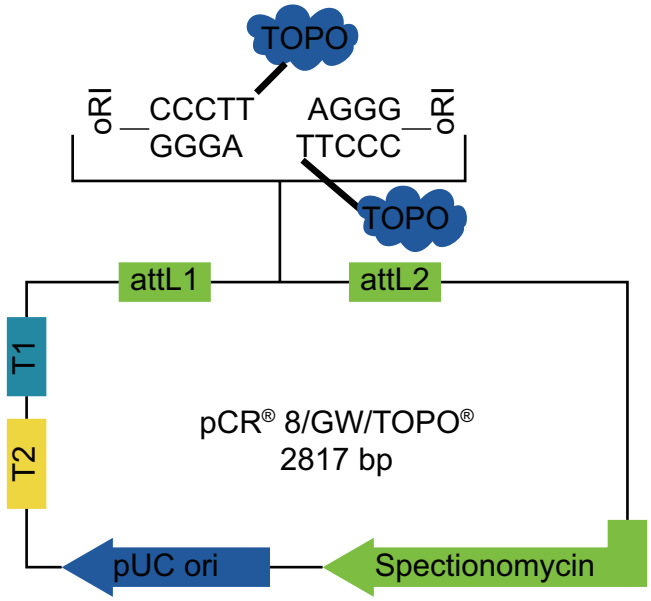

Figure 2.1 The vector map for pCR® 8/GW/TOPO®

pUC ori is the replicon site. T1 and T2 are transcription termination sequences. attL1 and attL2 are gateway clone sites for LR reaction. The small window open upward indicated the TOPO cloning site. The Spectinomycin is the SpnR gene enable spinctionomycin resistance. The is a M13 forward primer binding site near the attL1 site. Picture was derived from Life technology (Life Technologies).

2.3.2.5 Isolation of plasmids

Plasmids were isolated from the LB culture using Invisorb® spin plant mini kit (STRATEC Biomedical AG, Germany) with following manufactory's instruction. The isolated plasmids concentration and purity was measured by using a NanoPhotometer P300 (Implen, United States).

The correctness of the cloning was confirmed in three steps: colony PCR (described in 2.3.2.4, to assure the presence of a targeting sequence in desired direction), plasmid electrophoresis (checking the overall length of the plasmid to assure only one copy of targeting sequences is inserted in the vector), and DNA sequencing (to check the fidelity of the desired sequences). The expected size of the plasmid was the sum of the vector size and the targeted sequences, which is roughly confirmed by electrophoresis with the method described in section 2.3.2.2. An aliquot of the plasmid with expected length was sequenced with M13 forward sequencing primer (-20) (sequenced by Eurofins Genomics). The sequencing result was aligned with expected sequences. Plasmid with one copy of exactly the desired sequences in correct direction was ready for LR reaction.

2.3.2.6 LR rection to pHGWFS 7.0 vector

To transfer the promoter fragment into pHGWFS 7.0 vector (Figure 2.2), LR reaction with pCR® 8/GW/TOPO® vector carrying promoter fraction with pHGWFS 7.0 vector was

carrying out by using Gateway® LR Clonase® Enzyme mix, with instruction provided by Life technologies.

By following through the method described in section 2.3.2.4 and section 2.3.2.5, the resulting LR reaction mix was transformed to *E. coli* TOPO 10 competent cells, *E. coli* carrying the promoter fragment in pHGWFS 7.0 vector, was selected, confirmed, and propagated. Plasmids were isolated from the confirmed *E. coli* strains.

The isolated plasmid was transformed to *A. Tumefaciens* GV3101 (pMP90) competent cell using the freeze-thaw method (Weigel and Glazebrook, 2006). The competent cell are prepared according to the method described by Mellenthin (2012). The GV3101 (pMP90) contains a rifampicin resistance gene and a gentamicin resistance gene (Koncz and Schell, 1986). The pHGWFS 7.0 vector contains a spectinomycin resistance gene (Karimi et al., 2002). After transformation, the cells were transferred on the YEB solid medium (5 g l^{-1} Bacto Peptone, 1 g L^{-1} yeast extract, 5 g L^{-1} beef extract, 5 g L^{-1} sucrose, 1% agar, w/v) supplemented with 150 mg L^{-1} rifampicin and 25 mg L^{-1} gentamycin, and 100 mg L^{-1} spectinomycin. The plate was kept in a 28 ºC dark incubation chamber upside down for 2 days. The colonies come out were verified for the procession of cloned promoter sequences in desired direction with colony PCR (see section 2.3.2.4), using the forward primer designed binding immediately before attR1 site in pHGWFS7.0 vector and a corresponding reverse primer. After confirmation, 5 mL YEB (Yeast Extract Broth) medium (without agar, supplemented with 150 mg L^{-1} rifampicin and 25 mg L^{-1} gentamycin, and 100 mg L^{-1} spectinomycin) was inoculated with a single colony and grow for 48 h at 28 ºC. The overnight culture was stored in 15% sterile glycerol (v/v) and kept in -80 ºC refrigerator.

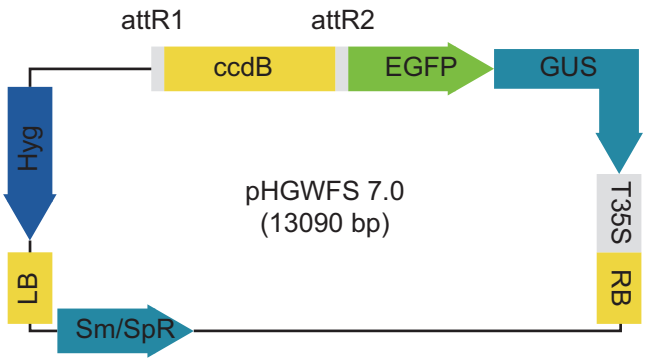

Figure 2.2 The vector map for pHGWFS 7.0

LB and RB are the left and right boarder of sequences that would be inserted in the plant genome. Hyg is the hygromycin resistant gent. EGFP-GUS cassette code for a fused protein of GFP and GUS. T35S are transcription termination sequences. attR1 and attR2 are gateway clone site for LR reaction. ccdB is lethal gene important for gateway cloning selection. The Sm/SpR gene enables spectinomycin resistance. Vector map was derived from Karimi et al. (2002).

2.3.2.7 Transformation of Arabidopsis

Arabidopsis was grown under short day conditions (10 h light/14 h dark regime, section 2.2.2) for 5 weeks. The plant was then transformed to long day conditions. After bolting, the major inflorescence was removed. The release of apical dominance by this way allows the development of more lateral florescence and greatly synchronized the flowing blooming time within a plant (Clough and Bent, 1998).

After the plant develops lots of flower buds, Agrobacterium was propagated for flora dip. Agrobacterium cells from -80 ºC stored stock were inoculated to 5 vials (20 mL in volume) containing 5 mL YEB medium supplemented with 25 mg L^{-1} gentamycin, and 100 mg L^{-1} spectinomycin. The overnight cultures were combined and pour to 500 mL Erlenmeyer flask containing 200 mL 150 mg L^{-1} rifampicin and 25 mg L^{-1} gentamycin, and 100 mg L^{-1} spectinomycin. The culture was further kept on a 27 ºC shaker bed for another day until the OD600 reached 0.8. The cells were then harvested by centrifugation for 8 min at 3000 ×g at room temperature (Eppendorf centrifuge 5810R, Germany). The supernatant was discarded. The pellet was suspended with activation buffer (5% sucrose and 0.05% surfactant Silwet L-77 (v/v. Warenzeichen Chemtura Corp., USA)).

The Arabidopsis inflorescences were infiltrated with activation buffer containing the Agrobacteria for 3 min using desiccator with a vacuum pump. The infiltrated plants was laid side down on a plastic tray. The tray was covered by a plastic bag to keep humidity. The tray was kept in dark at 20 ºC overnight. The plants were then grown in a greenhouse with the long day condition (see section 2.2.2) to propagate seeds. The seeds thus collected should contain transformed T1 seeds as well as un-transformed seeds. The expected transformation rate using this method is ab. 1%.

2.3.2.8 Transformant selection and propagation for homozygous lines

In order to select the transformed T1 plants from its un-transformed wild type counterparts, Hygromycin B resistance selection was used. 1/2 MS medium was poured on 12 cm rectangular Petri dishes and cooled on sterile bench until it solidified. The seeds from a single transformed plant (ab. 200 µL in volume) was sterilized and suspended in cooled above mentioned MS medium (ab. 60 ºC, felt by hand) and poured on the Petri dish. The Petri dish was then sealed. The seeds were stratified at 4 ºC for 2 days. 1 day exposure to 100 µmol photons m^{-2} s^{-1} white light was used for germination. Subsequently, the Petri dish was then covered by aluminium foil and kept in dark at 20 ºC for 4 days.

Those T1 seedlings with T-DNA had long hypocotyls and closed cotyledons while un-transformed had short hypocotyls and open cotyledons. The transformed T1 seedling were transferred to 1/2 MS medium without antibiotics for recovery of root growth and greening. 1 week later, the plants were transferred to soil and subjected to normal growth condition (defined in section 2.2.2) for seeds propagation. Each single T1 plant and its subsequent offspring here stand for an independent line carrying unique insertion in the genome.

Each T1 was propagated to the next two generations. The pedigree was recorded. The T2 generation was expected to have a Mendelian distribution of hygromycin resistance (25% homozygous plant hygromycin resistant, 50% heterozygous plant hygromycin resistant, 25 % wild type). The T3 offspring of those homozygous T2 plant were 100% hygromycin resistant, which is again verified by hygromycin resistance test described before. Besides, plants carrying more than one T-DNA insertion locus were not likely selected due to the propagation and selection. T3 homozygous plants were further used for other studies.

2.3.3 Confirmation of T-DNA lines

In order to study the role of putative binding transcription factors on controlling transcription of tAPX, several T-DNA lines corresponding to predicted binding transcription factor were purchased from ABRC (Arabidopsis Biological Resource Center). The gene numbers and T-DNA insertion line names are given here (Supplementary Data 2).

Seeds obtained from ABRC could be either homozygous or heterozygous for the T-DNA insertion of the required gene. The homozygosity was verified by three-primers-PCR with genomic DNA isolated from the lines. Two of primers are given in (Supplementary Data 2). The third one is a forward primer expected to bind to T-DNA sequences (LB1.3b primer with the sequence of ATTTTGCCGATTTCGGAAC for SALK lines, and SAIL-LB3 with the sequence of TTCATAACCAATCTCGATACAC for SAIL line). Homozygous lines should have one band with size of 400 - 700 bp; wild type should have one band with size of 900 - 1100 bp; heterozygous lines should have both the bands (Salk Insititue Genomic Analysis Laboratory, 2003). The homozygous T-DNA lines were used for further study. The heterozygous lines were propagated until homozygous offspring were obtained.

3 week old T-DNA lines as well as wild type plants were grow under normal short day conditions (Section 2.2.2). RNA was extracted to synthesis cDNA (Section 2.6.1). Using this cDNA as template, PCR using primer in (Supplementary Data 2) was performed to shown how much the gene of interest is knocked down.

2.4 Plant treatment

2.4.1 Light intensity treatment

Plants were put to 0-400 μmol photons m^{-2} s^{-1} light (L36W/840 Lumilux Cool White bulbs, OSRAM, Germany) conditions which in controlled by climate-controlled chamber. 1000 ± 200 μmol photons m^{-2} s^{-1} were provided by incandescent lamps.

2.4.2 Light quality treatment

Experimental plants were subject to irradiation of white light, dark, blue light, red light, and far-red light, for indication time (as described in Results section). White and dark conditions were performed in climate-controlled chamber with and without 50 μmol photons m^{-2} s^{-1} light. 50 μmol photons m^{-2} s^{-1} continuous blue light, red light, and far-red light irradiations were provided by mono-spectrum growth chamber (CLF PlantClimatics, Emersacker, Germany): blue light (LED, centroid at 471 nm), red light (LED, centroid at 673 nm), far red light (LED, centroid at 745 nm). Temperature of both the climate-controlled chamber and the mono-monochrome growth chamber, were kept at 22 ºC.

2.4.3 Temperature treatment

4 ºC and 30 ºC were used as cold stress and heat stress respectively. Plants were transfer to cold (climate-controlled chamber, 4 °C, 10 h 100 μmol photons m^{-2} s^{-1} light/14 h dark) or heat stress (climate-controlled Percival, 30 °C, 10 h 100 μmol photons m^{-2} s^{-1} light/14 h dark).

2.4.4 Drought treatment

3-5 weeks old soil grown plants were used for drought treatment. They were grown under normal light and temperature regime. Compared to control plants which were watered regularly, the drought treat plants were kept without watering for more than 3 days. The soil of the pots was totally dry.

2.4.5 Salt treatment

2 weeks old sterile cultured seedlings were used for drought treatement. Sterilized seeds were sown on the MS medium supplemented with 100 mM or 200 mM NaCl. The other growing procedure was the same with section 2.2.1.

2.4.6 Chemical treatement

25 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 2,5-Dibromo-6-isopropyl-3-methyl-1,4-benzoquinone (DBMIB), and N,N'-dimethyl-4,4'-bipyridinium dichloride (MV) were used for modification of the redox state of photoelectron transport chain. 1 mM H_2O_2 and 1 mM dithiothreitol (DTT) were used to modify the redox state and ROS level in plant cell. 1 mM IAA, 6-benzylaminopurine (BAP), abscisic acid (ABA), salicylic acid (SA), and jasmonic acid (JA) were used to study the plant phytohormone response. 25 μM lincomycin B (Lin) and norflurazon (NF) were used to disturb the chloroplast gene expression and carotenoid respectively.

Experimental plants were sprayed and infiltrated with indicated chemicals and subsequently transferred to 120 μmol photons m^{-2} s^{-1} light condition for 6 hours.

2.5 Reporter mesurement

2.5.1 GUS staining

The histochemical GUS staining was performed by incubating plant material in GUS staining buffer (1mM 5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid; 0.1% Triton X-100; 50 mM Na_2HPO_4/NaH_2PO_4 buffer, pH=7.2; 1 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6$) at 37 °C overnight and subsequently decolorized with 95% ethanol prior to observation (Jefferson et al., 1987). Picture of GUS stained plant material was taken by either a camera or stereoscopy.

2.5.2 Quantitative measurement of GUS activity

GUS activity was measured by monitoring the coulor change of β-glucuronidase catalyzing colorigenic convertion of the β-glucuronidase substrate p-Nitrophenyl-β-D-glucuronide (pNPG) (Gallagher, 1992). The assay was adapted so that large numbers of samples could be assayed and measured in a 96-well plate format. Plant tissues were collected into microcentrifuge tubes containing 1 or 2 glass beads (50 mm in diameter) and frozen in liquid nitrogen. Tissues were homogenized twice using a Retsch Ball Mill (Retsch GmbH, Haan, Germany) at a frequency of 30/s for each time 60 seconds. After homogenization, 200 μL GUS Extraction Buffer (50 mM sodium phosphate, pH 7.0; 10 mM β-mercaptoethanol; 10 mM EDTA and 0.1% Triton X-100) was added, and samples were mixed briefly on a votex shaker. The mixture was centrifuged at 13000 rpm for 15 min at room temperature in microcentrifuge

(Microcentrifuge 5424, Eppendorf, Germany). The supernatant was used for the colorigenic reaction. It was performed on a flat bottom 96 wells microtiter plate. For each sample, 10 µL of supernatant was mix with 90 µL GUS reaction buffer (50 mM sodium phosphate, pH 7.0; 5 mM DTT; 1 mM EDTA; 1.25 mM pNPG was added before use) or 90 µL GUS reaction buffer control (The recipe is the same with GUS reaction buffer without adding pNPG). The reaction tube were kept in 37 ºC for 120 min and stopped by adding 100 µL 50 mM $NaCO_3$. The absorbance at λ=405 nm indicating the color change was read by microtiter plate reader (Multiskan Spectrum, ThemoScientific Inc., Waltham, MA, USA). The GUS activity was calculated by the following equation.

$$\Delta\mathrm{Abs} = \mathrm{Abs}_{\mathrm{reaction}} - \mathrm{Abs}_{\mathrm{control}} \qquad \text{Equation 2.1}$$

$$\Delta c = \Delta\mathrm{Abs}/\lambda L \qquad \text{Equation 2.2}$$

$$E = \Delta c/t \qquad \text{Equation 2.3}$$

$Abs_{reaction}$: absorption of sample with reaction buffer; $Abs_{control}$: absorption of background (sample with control buffer); λ: molar extinction coefficient of the product of the reaction (4-nitrophenyl), with the value of 17800 M^{-1}, cm^{-1}; L: the height of final reaction buffer in microliter plate, which is 0.5 cm; c: the concentration of 4-nitrophenyl; t: time, 120 min.

GUS activity readout for each sample was normalized to the total protein content. Total protein content of GUS extraction was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, München, Germany), according to the manufacturer's instructions. 2 µL protein extraction was mixed with 200 µL diluted Bio-Rad buffer (1 part Bio-Rad buffer with 4 parts distill water) in 96 wells microtiter plate and incubated at room temperate for 5 min. The absorbance was readout by microtiter plate reader at λ=595 nm. Comparison to a standard curve performed with 0 ~ 4 mg/L bovine serum albumin (BSA) provided a relative measurement of protein concentration.

For each experiment treatment, all absolute GUS activity values were standardized as relative value to experimental control, whose value was arbitrarily set as 100%.

2.5.3 GFP measurement

GFP fluorescence was visualized by using a modular plant imaging system (NightSHADE LB 985, Berthold, Germany). Whole plants of 3-6 weeks old were put in the light-tight dark chamber. Excitation light was emitted by a halogen lamp (75 W, 340 - 750 nm, Philips). 10 s or longer excitation light was used. The excitation light was filtered by 480 nm filter. Florescence was given off by plant because of the GFP protein. The intensity of GFP is

correlated with the amount of protein, which is dedicated by the promoter activity in the experimental conditions. The emission light passes through a 530/25 nm filter. Images were taken with the NightSHADE Camera, using a 45 s exposure time, high gain, and a slow readout. Quantification was performed using IndiGO (version 2.0.3.0)

2.6 Transcript abundance analyses

2.6.1 RNA isolation

100 mg plant material was collected in 2 mL round bottom microcentrifuge tube supplemented with sterile glass bead and immediately immersed in liquid nitrogen. It was homogenized twice using a Retsch Ball Mill at a frequency of 30/s for each time 60 seconds. The following procedures were using GeneMATRIX Universal RNA Purification Kit (EURx, Poland) according to the manufactory's instruction.

2.6.2 RNA purity checks

NanoPhotometer P300 (Implen, United States) was used to measure the absorbance at 230, 260, 280 and 320 nm of the isolated RNA. Both A260/A230 absorbance ratio and A260/A280 absorbance ratio indicate protein contaminations. Only RNA samples with both ratios higher than 2.0 were used for the next steps. The RNA concentration was calculated by the implemented method of NanoPhotometer P300, which uses A260 and A230.

2.6.3 RNA integrity checks

The integrity of an RNA sample is determined by the integrity of ribosomal RNA. 10 µL RNA was mixed with 2 µL of 6× loading buffer (650 mM Tris-HCl, pH 6.8; 50% (v/v) glycerol; 0.025% (w/v) bromophenol blue). The mix was subjected to electrophoresis for 20 minutes at constant voltage (110 V) in 1 x TAE buffer. RNA with acceptable integrity is expected to give two clear bands, which indicate 28S and 18S ribosomal RNA (sometime a band for 5.8S ribosomal RNA is also visible, but not a request). Or else the sample electrophersis gives smears and are not taken for cDNA synthesis.

2.6.4 cDNA synthesis for gene expression analyses

cDNA was synthesized from isolated RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, United States) according to its protocol. Oligo (dT) primers was used in the cDNA synthesis process. For each reaction, 500 ng of RNA was used. The

resulting cDNA was diluted with 40 µL RNase-free water. Thus, the final cDNA concentration is 25/3 ng/µL.

2.6.5 Detection of genomic contamination of cDNA

The quantification of transcript abundance later on would be interfered by the presence genomic DNA in the cDNA sample. The confirmation that the cDNA is free of genomic DNA was conducted by RCR amplification of 2CPA gene (gene number) using a pair of primers that spanning an intron. The primers were OH1: 5'- GACTTTACTTTCGTCTGC-3' and OH4: 5'- ATCACTCCTTCCTTGTCG-3'. The PCR procedure and electrophoresis detection were similar with that descripted before. The contaminated sample would give a band with size of 585 bp in the electrophoresis detection. While genomic DNA free cDNA would give a band of approx. 350 bp and without the 585 bp bands. Only cDNA free of genomic DNA was used for qRT-PCR.

2.6.6 The primer design and verification

The primers (Table 2.4) used in qRT-PCR for this research were design previous by Juszczak (2013). The specificity was satisfying. All the primers have similar amplification efficiency around 1.

Table 2.4 The primers used in qR-PCR

Gene	Gene code	Name of the primer pair	Forward primer Reverse primer	Annealing temperature [°C]
Act2	At3g18780	qPCR_Act2	AATCACAGCACTTGCACCAAGC CCTTGGAGATCCACATCTGCTG	60
sAPx	At4g08390	qPCR_sAPx_1	AGAATGGGATTAGATGACAAGGAC TCCTTCTTTCGTGTACTTCGT	60
tAPx	At1g77490	qPCR_tAPx	GCTAGTGCCACAGCAATAGAGGAG TGATCAGCTGGTGAAGGAGGTC	60
2CPA	At3g11630	qPCR_2CPA	CCCAACAGAGATTACTGCCT ATAGTTCAGATCACCAAGCCC	60

2.6.7 qRT-PCR

Transcript abundance of selected genes was quantified by qRT-PCR, which was performed according to the MIQE guidelines (Bustin et al., 2009). For each reation, a total volume of 20 µLPCR components (see Table 2.5) was added on Hard-Shell® 96-Well 480 PCR Plates. SYBR Green I (Sigma-Aldrich Chemie GmbH, Munich, Germany) used in the reaction is able to bind DNA. The resulting DNA-SYBR Green I complex absorbs blue light (λmax = 497 nm) and emits florescence (λmax = 520 nm). CFX96 thermocycler (BioRad, United States) was used for PCR amplification and monitoring florescence. Table 2.6 gives the amplification and monitor schedule. Florescence detection was according to the preset of thermocycler. Fluorometrical determination in the cycling phase would give sigmoid curve, the shape of which is correlated with the template abounded and important for threshold cycle (Ct) calculation. The lateral fluorometrical determination results a melting curve useful for primers specificity inspection.

Table 2.5 Component of qRT-PCR reaction

Substance	Volume (µl/well)
10× Buffer[a]	2.00
50 mM Magnesium Chloride	0.80
5 mM dNTP[b]	0.40
10× SYBR Green I	0.20
Water	13.44
5 U/µl OptiTaq-Polymerase	0.04
50 µM Primer mix[c]	0.12
cDNA Template	3.00
Final Volume	20.00

Table 2.6 qRT-PCR amplification and monitoring

Steps		Temperature	Time
Pre-denaturartion		95 ºC	10 min
40 cycles	Denaturation	95 °C	15 sec
	Annealing	60 °C	30 sec
	Fluorometrical determination		
	Extension	72 °C	30 sec
	Fluorometrical determination		
Post-denaturartion		95 °C	10 sec
Melting curve test[d]		60 : 95 °C by 0.5 °C	5 sec

Note:

a: 10× Buffer is composed of 160 mM ammonium sulfate, 1 M Tris-HCl, pH 8.3, and 0.10% Tween-20 (v/v); b: The mixture is composed of 5 mM dATP, 5 mM dCTP, 5 mM dGTP and 5 mM dTTP; c: a mixture of 5 µM forward primer and 5 µM Reverse primer for studied gene. d: A sequence of temperature from 60 to 95 ºC, with a incensement of 0.5 ºC, fluorometrical determination at each temperature step.

The relative quantity was calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). In $2^{-\Delta\Delta Ct}$ equation, ΔCt corresponds to the difference in Ct values between the gene of interest and gene used for normalization (in this study it is Act2, AT3G18780), whereas ΔΔCt is the difference in ΔCt between the analyzed and experimental control.

For each sample in the transcript analysis experiment, 3 biological replicates and 3 technical replicates were used. Biological replicates referred sampling plant material with sample treatment and genetic background. The technical replicates indicated 3 replications in qRT-PCR reactions. The relative expression value, *i.e.* the expression ratio of the treatment and control was used. The expression value for the control in the experiment was thus automatically set as "1".

2.7 ROS detection

2.7.1 DAB staining of H_2O_2

To visualize H_2O_2 *in situ*, 3, 3'-diaminobenzidine (DAB) staining was performed on Arabidopsis leaves. H_2O_2 oxidizes DAB and produces an easily observable brown color. Leaves were vacuum-infiltrated with 1 mg/mL DAB in 1x PBS (73 mM sodium chloride, 3 mM potassium chloride, 10 mM disodium hydrogen, and 2 mM monopotassium phosphate) and kept in dark chamber at room temperature (approximately 8 h). Subsequently, the leave

material was de-chlorophyllied by incubation in 96% ethanol (v/v) overnight. Pictures were taken after the green color was totally removed.

2.7.2 Quantitative peroxide assay

A method similar with Pierce™ quantitative peroxide assay was used to quantify the H_2O_2 in plant tissue (reference). In the presence of acidic environment, Fe^{2+}, and sorbitol, H_2O_2 oxidizes xylenol orange dye (o-cresolsulfonephthalein-3′-3′-bis-(methyliminodiacetic acid sodium salt), Sigma-Aldrich, Germany) to yield a purple product having maximum absorbance at λ=560 nm.

Before the use, 1 volume of Reagent A (25mM ammonium ferrous (II) sulfate, 2.5 M sulfuric acid) was mixed with 100 volumes of Reagent B (100 mM sorbitol, 125 µM xylenol orange) to make working reagent. Approximately 100 mg fresh material was harvested, weighted and homogenized as method descripted before. 200 µL potassium cyanide (0.5 mM) was added to extract H_2O_2. After a brief shaking on vortex, the extraction was centrifuged (centrifuge 5415R, Eppendorf, Germany) for 10 minutes at 16100 ×g and 4 °C. 20 µL supernatant was added to 200 µL working reagent on 96 wells flat bottom microtiter plate, and incubated for 20 min at room temperature. The absorbance at λ=595 nm was read in microtiter plate reader (Multiskan Spectrum, ThemoScientific Inc., Waltham, MA, USA). The comparison of the readout for sample and H_2O_2 standard solution (concentration ranged from 0 to 200 nM) gave the H_2O_2 concentration.

3 Results

3.1 *in silico* study of *sAPX* and *tAPX* expression

3.1.1 Microarray of *sAPX* and *tAPX* in tissues, developmental stages, physiological and genetic perturbations

Microarrays provide enormous amount of expression data on different physiological conditions and genetics background. Genevestigator (http://www.genevestigator.ethz.ch/) is a web tool retrieving data from *Arabidopsis* Affymetrix GeneChip database (Zimmermann et al., 2004; Zimmermann et al., 2005). Using Genevestigator *Plant Biology* module, Data for probes 255142_at (*sAPX*, at4g08390) and 259707_at (*tAPX*, at1g77490) were retrieved from ATH1:22k array platform. The tissue specific expression was studied (**Figure 3.1**).

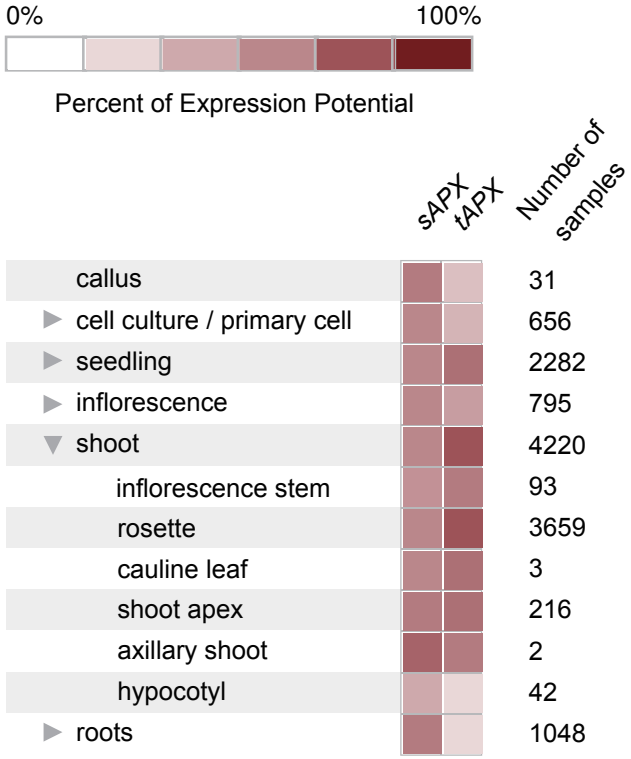

Figure 3.1 The expression pattern of *sAPX* and *tAPX* in different tissues. The white-red colors indicate the absolute values of gene expression, with the white representing the lowest expression and the darkest red standing for the maximum expression. The expression of *sAPX* is highest in roots and lowest in hypcotyls. The expression of *tAPX* is highest in rosette and lowest in roots. The number after the color bricks indicate how many different microarray data were used for the data in Genevestigator.

Figure 3.1 represents *sAPX* and *tAPX* expression in absolute values, hence it is possible to compare the two genes' expression within the same tissue/organ. As shown in Figure 3.1, *sAPX* expressed is higher in callus and cell culture than *tAPX*, while *tAPX* expression in plant

seedling and adult rosette is higher. *sAPX* expression in roots is comparable with that in leaves. Moreover, both *sAPX* and *tAPX* expression can be detected in the inflorescence. The developmental regulation of *sAPX* and *tAPX* has been studied by Panchuk et al. (2005) using RT-PCR showed that *tAPX* mRNA abundance is decreasing with leaf senescence, while *sAPX* is not regulated by development. Perturbation analysis was performed in Genevestigator, to distinguish factors changing *sAPX* and *tAPX* expression based on previous microarray studies (Supplementary Data 3). There were 3072 individual perturbation experiments listed at the time point of analysis (Checked from 2014, January). Each experiment had at least 2 replicates. Using changing fold ≥2, *p*-value ≤0.001 as criteria, 39 and 86 conditions change *sAPX* and *tAPX* expression, respectively (Supplementary Data 3-A, and B). Processes and conditions such as germination, callus formation, exogenous sugar, cold, disturbance of proteasome promote *sAPX* expression, while hypoxia, ion deficient, light extension suppress *sAPX* (Supplementary Data 3). Perturbations inducing *tAPX* expression include brassinosteroid application, far red light, mutations in light signal transduction pathways. *tAPX* suppression conditions are cold, lincomycin B, norflurozon treatment, mutations in brassinosteroid signal transduction pathways, mutants in light signaling, drought, *etc.* Noticeably, there are differences of *tAPX* expression among ecotypes which are Arabidopsis accessions from different geographic origins.

3.1.2 Co-expression genes with *sAPX* and *tAPX*

The arrangement of functionally distinct genes in the one regulation group facilitates the co-ordination of different functions to sustain plant growth and development. The transcriptional regulation patterns of *tAPX* and *sAPX* are clustered with other nucleus encoded chloroplast genes, most of which have distinct roles other than ROS scavenging (Table. 3.1).

Table. 3.1 Top 10 co-expression genes with chloroplast ascorbate peroxidases

Data were retrieved from Gene Co-expression Analysis Toolbox (http://genecat.mpg.de/). Pearsons correlation coefficient (-1.0~1.0) determines the strenght and direction of a linear relationship between any two variables. The localization is based on SUBAcon from SUBA3 (Tanz et al., 2013).

	gene ID	Pearsons correlation coefficient	Annotation	Localization
			***tAPX* (At1g77490)**	
1	At5g36790	0.94159	putative phosphoglycolate phosphatase	plastid
	At5g36700		putative phosphoglycolate phosphatase	plastid
2	At1g09340	0.93602	expressed protein	plastid
3	At3g63140	0.93446	putative mRNA-binding protein	plastid
4	At1g75690	0.93220	chaperone protein dnaJ-related	plastid
5	At1g32080	0.93091	putative membrane protein	plastid
6	At4g09010	0.93046	putative chloroplast L-ascorbate peroxidase	plastid
7	At5g38520	0.92886	hydrolase	plastid
8	At3g48420	0.92769	haloacid dehalogenase-like hydrolase family protein	plastid
9	At4g39970	0.92611	haloacid dehalogenase-like hydrolase family protein	plastid
10	At3g55800	0.92607	chloroplast enzyme sedoheptulose-1,7-bisphosphatase	plastid
			***sAPX* (At4g08390)**	
1	At1g53240	0.64296	malate dehydrogenase (NAD)	mitochondrion
2	At2g33210	0.63867	putative chaperonin	mitochondrion
3	At3g47520	0.63508	malate dehydrogenase (NAD)	plastid
4	At4g37910	0.62202	heat shock protein 70	mitochondrion
5	At4g24830	0.59167	arginosuccinate synthase family protein	plastid
6	At5g14040	0.58275	mitochondrial phosphate transporter	mitochondrion
7	At1g23100	0.57270	10 kDa chaperonin, putative	mitochondrion
8	At4g10480	0.57221	nascent polypeptide associated complex alpha chain proteins	cytosol
9	At4g26210	0.56339	mitochondrial ATP synthase g subunit family protein	mitochondrion
10	At2g20420	0.56194	succinyl-CoA ligase (GDP-forming) beta-chain	mitochondrion

The transcriptional regulation of *tAPX* and *sAPX* can be inferred from the former study of other genes in the same cluster. For instance, *tAPX* might have the same light/dark regulation pattern like its co-expression partner, the chloroplast enzyme sedoheptulose-1, 7-bisphosphatase (Willingham et al., 1994; Hahn et al., 1998) (Table. 3.1). Systematic study on one gene also shed light on how other regulates within the co-expression list are regulated. *tAPX* is clustered with other chloroplast targeted genes with high Pearsons correlation coefficient, while *sAPX* has no closely coregulated genes (the highest Pearsons correlation coefficient for *sAPX* co-regulated gene is 0.643). Futhermore, the top 10 co-expression genes for *tAPX* are all coding for plastid localized proteins while for *sAPX* are coding for plastid, mitochondrion, and cytosol localized proteins. There indicated *sAPX* and *tAPX* are differently regulated despite their similar physiological functions.

3.1.3 Promoter *cis*-element analysis of *sAPX* and *tAPX*

cis-regulatory elements are comprised of 4-20 non-coding DNA sequences, which regulate the genes nearby. The binding of transcription factors to the corresponding *cis*-acting regulatory DNA element determines the activation or repression of genes. To obtain an insight of how *sAPX* and *tAPX* are regulated transcriptionally, the *in silico* analysis of *sAPX* and *tAPX* upstream 2000 bp promoter were performed by PLACE (Higo et al., 1999), Athena (O'Connor et al., 2005), and AGRID Atcis DB (Davuluri et al., 2003). The binding motifs presented in at least two databases are listed in Supplementary Data 5-A, and B (for *tAPX* and *sAPX* respectively). Athamap (Hehl and Bulow, 2014) is a good tool for visualizing transcription factor binding sites, the schematic diagram of TF and the transcription binding site are addressed below (Figure 3.2).

There are 11 different *cis*-motifs, and 16 different *cis*-motifs found in the *tAPX* and *sAPX* promoter region, respectively. The most distinct feature of *tAPX* is that it is rich in development regulating motifs. AGATCONSENSUS, CARGCW8GAT, LEAFYATAG found in the *tAPX* promoter are motifs regulating the transcription of AGAMOUS or AGAMOUS-like genes, which are C-function floral organ identity genes. Besides, RAV1-B regulates AP2-like and B3-like domains protein RAV1, which might have certain function in rosette development (Hu et al., 2004; Woo et al., 2010). The embedding of motifs of development regulating genes in the *tAPX* promoter indicates that this chloroplast ascorbate peroxidase can be regulated by development, which associates the chloroplast development and leaf greening. There are other light regulating, phytohormone regulating motifs, as well. For the *sAPX* promoter, it is most striking that it enrichs MYC, MYB, and WRKY

transcription factors binding motifs (Iwasaki et al., 1995; Abe et al., 1997). These transcription factors are active in response to plant stress, specifically salt and osmotic stress, and abscisic acid. Other light regulating, stress regulating motifs are also seen.

There are four shared motifs by *sAPX* and *tAPX*, ARF1 (TGTCTC), RAV1-B (CACCTG), TBOXATGAPB (ACTTTG), and MYB1AT (WAACCA). This exemplifies that *sAPX* and *tAPX* can be transcriptionally regulated in the same way by the corresponding transcription factors. ARF1 and MYB1AT are phytohormone responding motifs, activated by auxin and abscisic acid respectively (Abe et al., 2003). RAV1-B is bound by RAV1 protein which contain AP2-like domain. TBOXATGAPB is a light activation motif. Interestingly, it is also found in the promoter of B subunit of chloroplast glyceraldehyde-3-phosphate dehydrogenase (GADPH) of Arabidopsis (Chan et al., 2001b). This indicates that the two chloroplast APX are uniformly regulated with other two nucleus encoded chloroplast proteins under certain conditions.

The transcription factor binding sites map illustrated in Figure 3.2 indicates that in *sAPX* and *tAPX* promoters the putatively binding sites are evenly spread in the promoter region. The majority of the binding motifs are C2C2 (Zn) Dof transcription factor binding motifs (5'-AAAG-3') for both *sAPX* and *tAPX*. There is preference for miRNA binding sites in the *sAPX* promoter according to the prediction. However, the miRNA regulation lies in the posttranscriptional phase, where the promoter is not taking part in. Thus, it is doubtful whether the two miRNA binding islands in the *sAPX* promoter are of relevance.

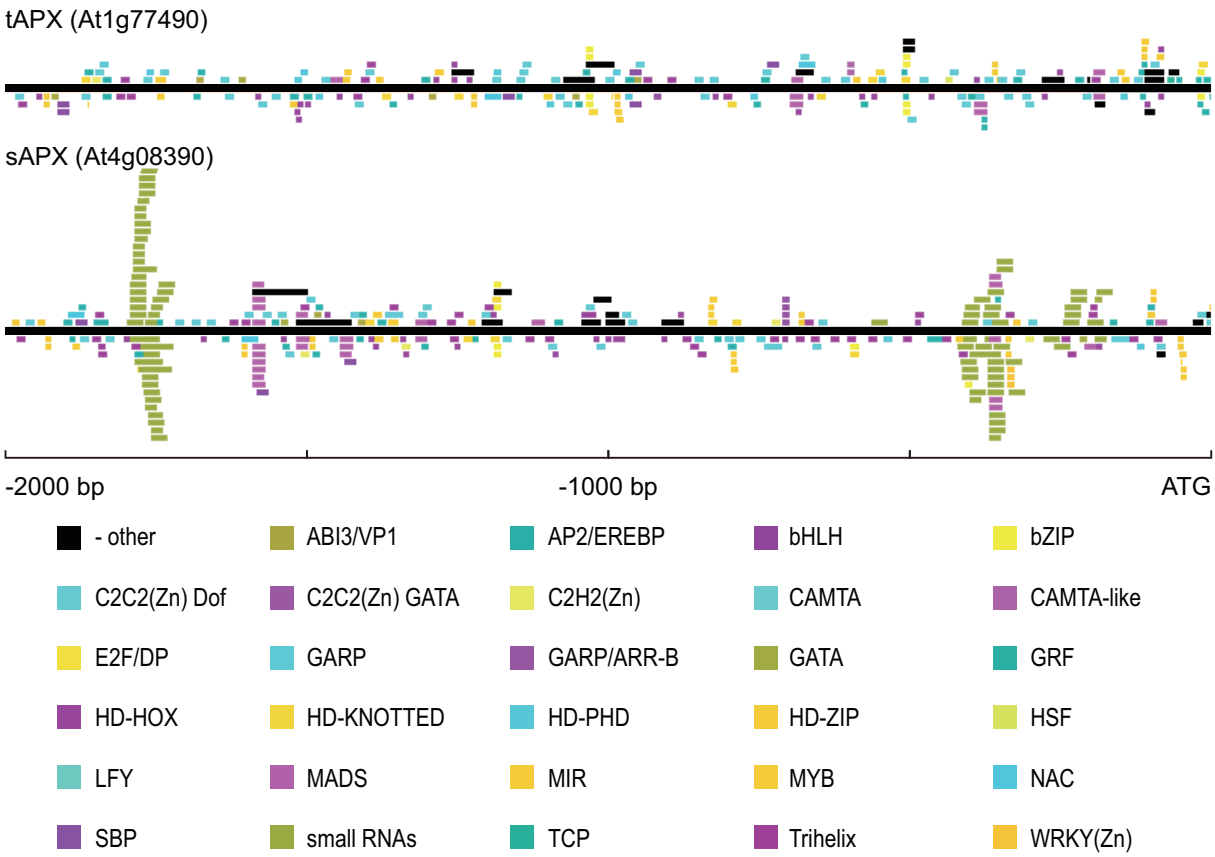

Figure 3.2 The expression pattern of sAPX and tAPX in different tissue. The two thick line above schematcally reprent the tAPX (upper) and sAPX (lower) promoters upstream of start codon ATG. The blocks represent transcription factors. Putative transcription factor binding sites are demonstrated along the promoters. The color legend for different codons is listed in the lower part (details of the putative transcription factors and their abbreviation is available on Athamap (http://www.athamap.de/)).

3.2 *In planta* analysis of *sAPX* and *tAPX* promoters

3.2.1 Promoter-reporter construction of *sAPX* and *tAPX*

Following the cloning and transformation procedure performed by U. Ellersiek and M. Baier, T3 homozygous plants carrying *pro*sAPX:EGFP-GUS and *pro*tAPX:EGFP-GUS were obtained. For each construct, 3 independent lines showing similar reporter activity were used for further study.

3.2.2 Tissue specific expression of *sAPX* and *tAPX*

Reverse genetic studies demonstrated the loss of function of sAPX and of tAPX causes differences in the phenotypes of T-DNA insertion lines (Kangasjärvi et al., 2008; Maruta et al., 2010). Given that sAPX and tAPX are highly conserved in protein sequences (Supplementary Data 5), and have nearly identical enzymatic properties (Table 1.1), the

difference in phenotype may originate from their sub-cellular localization (tAPX in chloroplast thylakoid, sAPX in chloroplast stroma and mitochondrion matrix; see section 1.2.5) and their gene expression difference. Using the reporter gene lines, the *sAPX* promoter was demonstrated to be less homogenous active in plant tissue than the *tAPX* promoter (Figure 3.3). The majority of the expression was seen in leaf and root vasculature (Figure 3.3 A, C, and E). The unevenness of the *sAPX* expression may be caused by different light conditions, raised from shading of leaves by one another. However, *tAPX* are more ubiquitously expressed in leaf tissues (Figure 3.3 B, D, and E). This explains why *tAPX* other than *sAPX* is demonstrated as the major antioxidant dealing with oxidative stress in mature leaves (Kangasjärvi et al., 2008; Maruta et al., 2010).

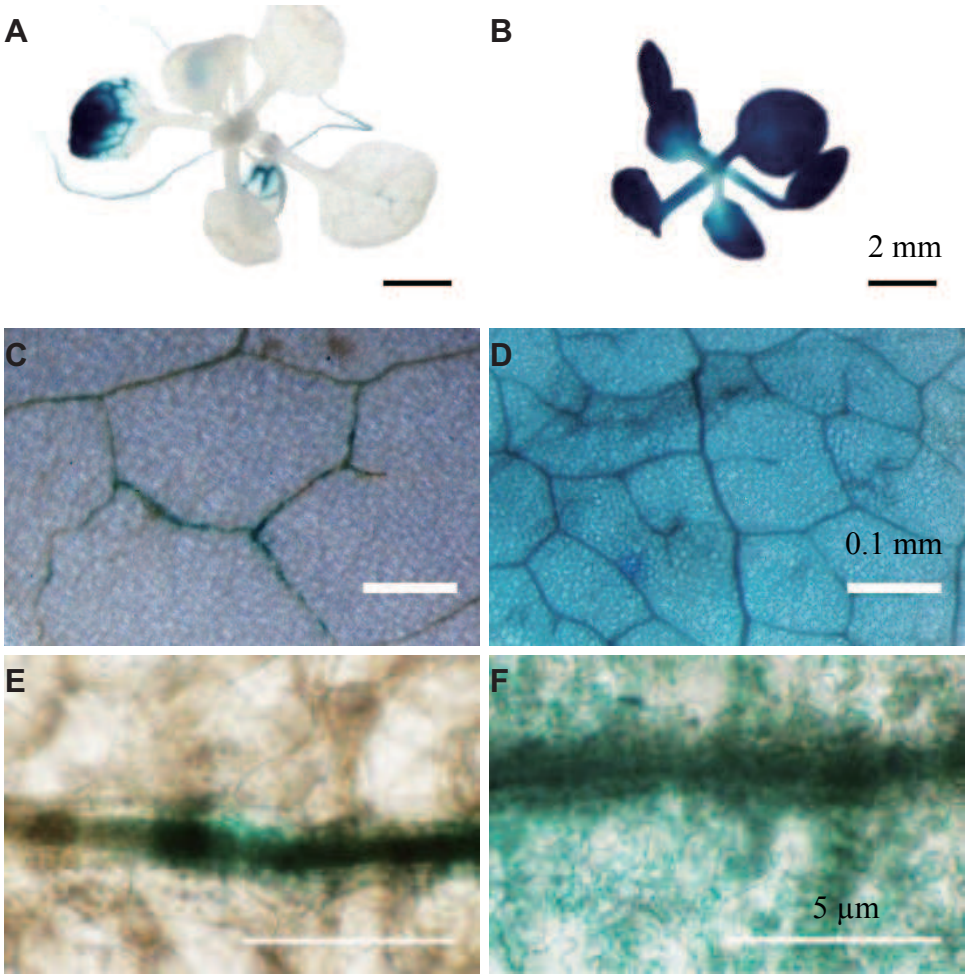

Figure 3.3 Tissue specific promoter activities of the *sAPX* and *tAPX*. T3 plants carrying p*ros*APX:GFP-GUS (A, C, E) and p*rot*APX:GFP-GUS (B, D, F) were grown on MS medium under controlled conditions (120 µmol photons m^{-2} s^{-1} white light, 22 °C/14 h dark, 18 °C). GUS staining was performed 3 weeks after sowing plant.

3.3 Transcriptional regulation of *tAPX*

3.3.1 Transcriptional regulation of *tAPX* by photosynthetic electron transport chain

Chloroplasts are the major locations for ROS production (Asada, 2006). The accumulation of H_2O_2 occurs especially when electrons generated in PET and downstream consumption, such as in the Calvin cycle and nitrate reduction is uncoupled. The redox states of PET components are shown to be able to regulate nucleus encoded chloroplast genes, including 2CPA (Pfannschmidt et al., 2001; Heiber et al., 2007; Shaikhali et al., 2008). It is tempting to propose that chloroplast ascorbate peroxidase genes are also controlled by PET. In order to elucidate the effect of PET on the regulation of *sAPX* and *tAPX* promoters, the reporter gene lines were tested for the response to different herbicides blocking photoelectron transport chain (Figure 3.4 A). Under light illumination, DCMU blocks electron flow at the quinone acceptors site of photosystem II, by competing for plastoquinone binding site (Ashton, 1973). Thus, the Q_B accepts fewer electrons from the photosystem II, which results an oxidized plastoquinone pool. DBMIB is a plastoquinone analogue which binds to the Q_0 site in the Cytochrome b_6f (Cyt b_6f). Complex and blocks electron transport downstream of plastoquinone pool (Chain and Malkin, 1979). An application of DBMIB generates a reduced PQ pool. Methylviologen (MV) effectively competes with $NADP^+$ as a PSI electron acceptor (Hatzios et al., 1980). Plants treated with MV accumulate ROS and draw the pool of electron acceptors downstream PSI to a more oxidized state in chloroplast.

When electron transport was blocked upstream of photosystem I (DCMU or DBMIB treatment) showed consistent repression effect of *tAPX* promoter, while the electron blocker downstream of photosystem I (MV treatment) showed an induction effect (Figure 3.4, B and C for upstream PSI; D for downstream PSI). An over 1.5 fold increase of GUS activity was seen in the MV treated plants. If *tAPX* is regulated the redox pool of PQ, the block of electron transport upstream (DCMU treatement) and downstream (DBMIB treatment) would have opposite effects on *tAPX* promoter. Instead, opposite effects on *tAPX* promoter were seen from block at Q_0 site of Cyt *b6f* and block at $NADP^+$. Therefore, electron acceptor in between (Cyt *b6f*, plastocyanin, PSI, Fd, and Fd-NADP reductase). Alternatively, *tAPX* promoter activity was triggered by chloroplastic H_2O_2, as MV treatment renders chloroplastic H_2O_2 accumulation.

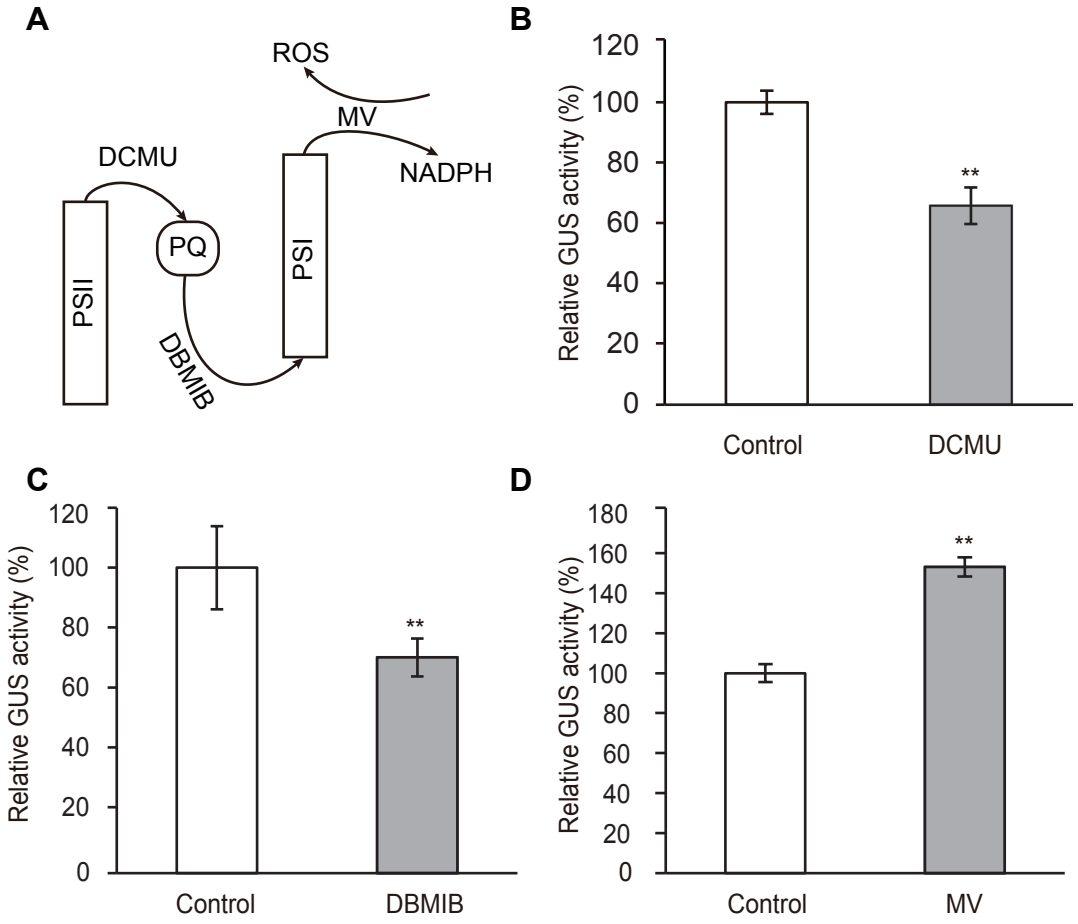

Figure 3.4 The photosynthetic electron transport effect on *tAPX* expression. (A) The block sites of photosynthetic electron transport chain by DCMU, DBMIB, MV. (B)(C)(D) GUS activity of *pro*tAPX:GUS-GFP lines (3 weeks old) treated with 25 µM DCMU (B), 25 µM DBMIB (C), and 25 µM MV (D). n≥6, ** indicates significant difference, p≤0.01.

3.3.2 *tAPX* promoter responses to wounding.

In order to connect the *tAPX* promoter activity to ROS levels in plant cells, mechanical injures to plant leaves was used. Mechanical wounding triggers a rapid and massive elevation of ROS in plant cells, because of the induced activity of plasma membrane localized NADPH oxidase (Orozco-Cardenas et al., 2001; Kumar et al., 2007). Hence, wounding triggered H_2O_2 is mainly localized to apoplast and cytosol. Wounding induction of *tAPX* promoter driven GUS expression was seen in young leaves, as suggested by the enhanced GUS staining along the marks caused by forceps-crashing (Figure 3.5 A and B). Forceps-crashing injuring induces ROS in both young and old leaves (Figure 3.5 C). However, *tAPX* promoter responses were only seen in young wounded leaves, but not in the old ones, indicating that the *tAPX* response to wounding is at least not directly caused by ROS elevation in the apoplast and cytosol.

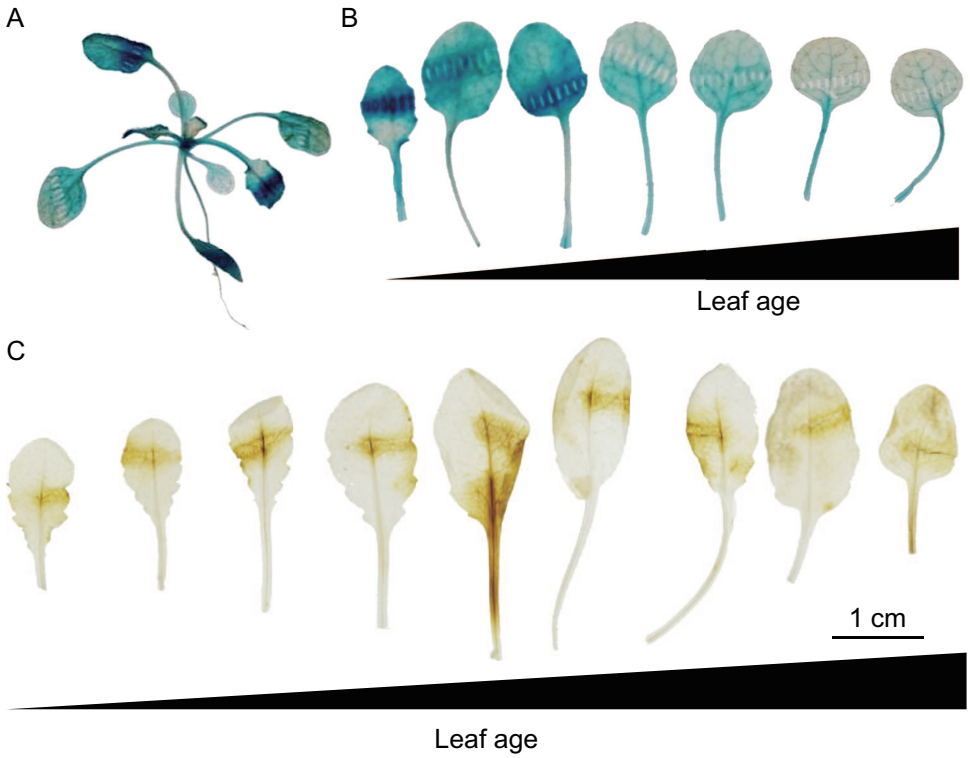

Figure 3.5 Mechanical wounding induces *tAPX* promoter. (A) 4 weeks old *pro*tAPX:EGFP-GUS plant were injured by forceps, GUS staining was followed 1 hour after forceps-crashing. (B) Leaves from (A) were dissected and arranged in age increasing manner (indicated by black wedge). (C) DAB staining indicated that wounding caused H_2O_2 production in leaves of all ages.

3.3.3 *tAPX* promoter is not regulated by cytosolic H_2O_2

To explain why *tAPX* promoter was not seen up-regulated by wounding in old leaves, that *tAPX* promoter is not regulated by cytosolic H_2O_2 was proposed. To verify this, various cytosolic H_2O_2 triggering stress conditions (high light, cold, drought, heat, and salt) and cytosolic H_2O_2 disturbing chemicals (DTT, H_2O_2, JA, SA, and ABA) were applied. If the assumption is correct, a negative result (no triggering effect on *tAPX* promoter) would be seen. The regulation of chloroplast antioxidant enzymes, such as 2CPA, is shown to be distinct from that of cytoplasmic antioxidants (Heiber et al., 2007). A cytoplasmic isoform of ascorbate peroxidase *APX2* promoter responds to very high level of H_2O_2 in the cytosol (Fryer et al., 2003). The expression of chloroplast antioxidant enzyme 2CPA only responds to more moderate stress (Heiber et al., 2007). The transcriptional regulation is mediated by Rap2.4a in a redox sensitive manner (Shaikhali et al., 2008). The following experiment would also enable the comparision of *tAPX* promoter regulation with that of *2CPA* and *APX2*.

Those environmental cues (Figure 3.6) are known cytosolic ROS triggers. The measure of GUS activity demonstrated that *tAPX* does not respond to 1000 µmol photons m^{-2} s^{-1} high

light. The exposure of plant to high light leads an overproduction of ROS (Havaux and Niyogi, 1999). The H_2O_2 regulating genes, such as *APX2*, are elevated in promoter activity upon such excess light (Fryer et al., 2003). The inertness of *tAPX* promoter activity to 1000 µmol photons m^{-2} s^{-1} high light excludes a similar regulation pattern of APX2, which was shown to be regulated by cytosolic H_2O_2 (Figure 3.6 A). The other cellular H_2O_2 inducing conditions tested were salt stress and drought stress. Except heat stress, which did not change *tAPX* promoter activity, the tested H_2O_2 inducing environments suppress *tAPX* expression (Figure 3.6 B-D). Feeding plants with H_2O_2 repressed *tAPX* promoter activity (Figure 3.6 E). Significant drop of *tAPX* activity was seen when plants were sprayed with 1 mM H_2O_2. On the contrary, addition of DTT (1 mM) was intended to induce a more reduced cellular redox state. Unexpectedly, reduction of *tAPX* promoter activity was also seen. This might be due to the inhibition effect of this strong reducing agent to signal transduction pathways as well as to other cellular components. For instance, the *2CPA* promoter is activated by the Rap2.4a transcription factor under certain redox poise condition, nevertheless, both strong reducing and oxidizing environment (provided by 5 mM DTT, and 5 mM H_2O_2 respectively) abolish the binding (Shaikhali et al., 2008). As noticed by Shaikhali et al. (2008), *tAPX* expression reduced in the T-DNA knock out lines of Rap2.4a. It is possible that *tAPX* is also regulated by Rap2.4a. If that is the case, the cellular redox state for optimal binding however is not equal to that of *2CPA*, since 1 mM H_2O_2 increases the *2CPA* promoter driven expression.

Besides, the H_2O_2 inducing phytohormones, such as jasmonic acid, salicylic acid, and abscisic acid, were applied to *protAPX*:EGFP-GUS plant. These hormones are known to cause cytoplasmic H_2O_2 accumulation by activation of the plasma membrane localized NADPH oxidases (Kwak et al., 2003; Hung et al., 2006; Kalachova et al., 2013). H_2O_2 in turn is important mediator for the phytohormone signaling pathways (Orozco-Cardenas et al., 2001; Hung et al., 2006; Kalachova et al., 2013). The test of phytohormone effect would provide an insight whether *tAPX* is regulated by cytosolic H_2O_2. Not a consistent reaction of *tAPX* promoter was seen among the above three H_2O_2 inducing phytohormone treated plant (Figure 3.6 F). Overall, the results here clearly suggest that *tAPX* promoter is not directly modulated by cytosolic H_2O_2 level.

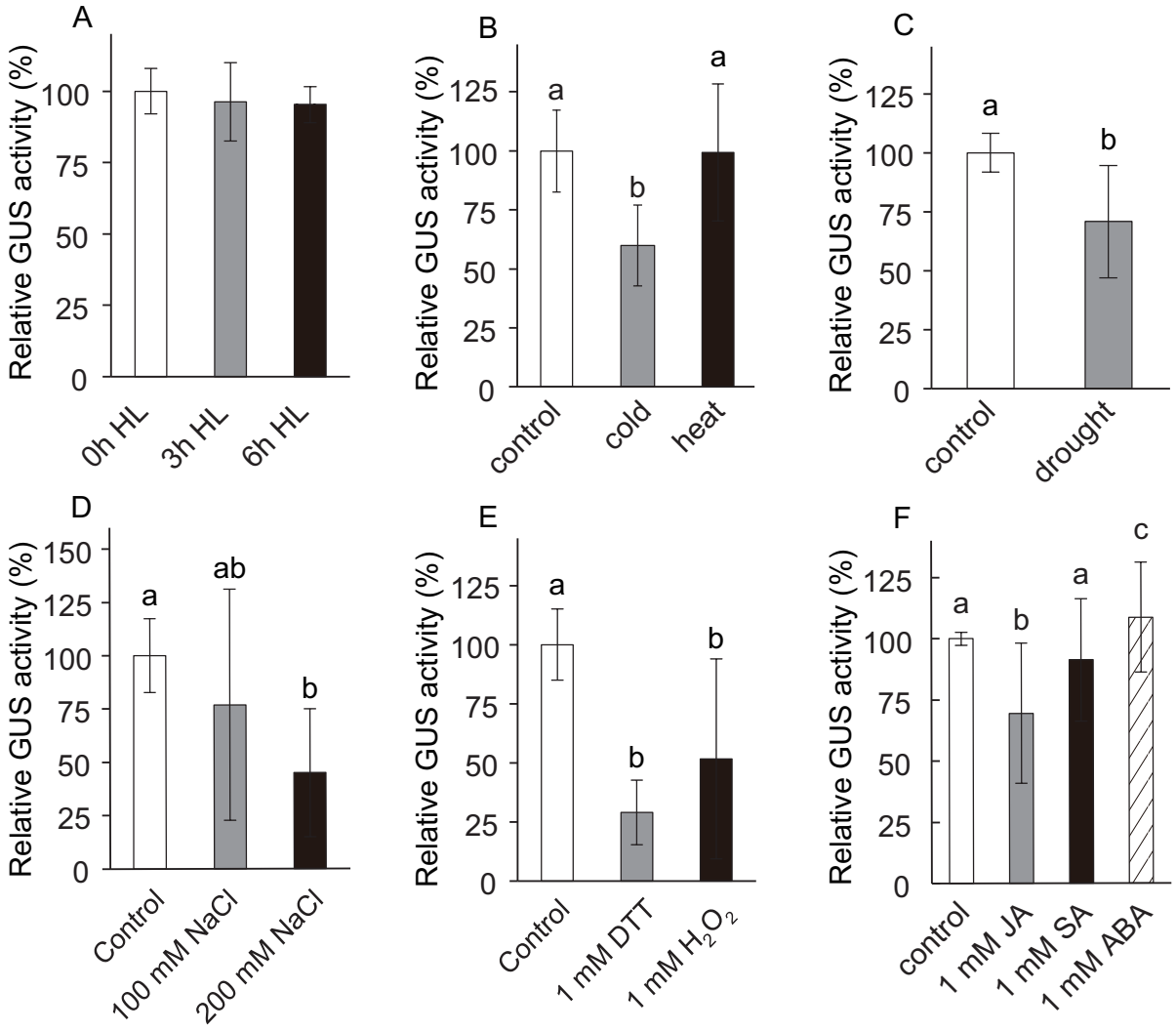

Figure 3.6 Effect of hydrogen peroxide inducing factors on tAPX promoter activity. *pro*tAPX:EGFP-GUS were grown on 1/2 MS media for 3 weeks (A-E) or on soil under normal growth condition. GUS activity were measured 0, 6, or 12 hours after transfer from 120 to 1000±200 µmol photons m^{-2} s^{-1} light condition (A); 12 hours 4 ºC (cold), and 30 ºC (heat) condition (B); and in drought (C); 48 hours after transfer to 1/2 MS media supplemented with 100 and 200 mM NaCl (D); 3 hours after spraying of the aerial parts with 1 mM DTT or H_2O_2 (E); 3 hours after spraying of the aerial parts with 1 mM JA, SA, and ABA (F). Data refer to the average of normalized 3 independent T3 transformation lines × biological replicates (±SE); different letters indicate pronounced difference, Student's t-test, $p \leq 0.05$.

3.3.4 *tAPX* promoter is regulated by chloroplastic H_2O_2

Data presented in section 3.3.3 unequivocally demonstrate that *tAPX* is not regulated by cytosolic H_2O_2. However, it would be beneficial for plants if the transcript of *tAPX* is coordinately fine-tuned in order to meet the antioxidant requirement, *i.e.* the chloroplastic H_2O_2 content, which is superior to a system depending on constantly expressing *tAPX* without adjustment. Possibly, chloroplastic H_2O_2 rather than cytosolic H_2O_2 carries out the

coordination with a transformed yet unknown mediating signal molecule(s) before diffusing to the cytosol. To test this hypothesis, additional experiments were conducted. Transferring the dark adapted (24 hours, to silence the photosynthesis related factors but not inducing chloroplast morphological change) plants to 100 μmol photons m^{-2} s^{-1} or 400 μmol photons m^{-2} s^{-1} activated the *tAPX* promoter driven GUS expression (Figure 3.7 A and B). The reason for the induction of *tAPX* promoter activity could attribute to the accumulation of H_2O_2 in "unprepared" chloroplasts as caused by the immediate transferring. The sudden exposure of plant to light and the chloroplastic antioxidant deficient modulate the H_2O_2 level and thus induce the tAPX level (Fryer et al., 2003). Mutants affect the H_2O_2 level inside the chloroplast provide a tool to study the chloroplastic enclosed H_2O_2 effect on the tAPX transcriptional regulation. In the *2cpb*, *2cpa2cpb* mutants, H_2O_2 accumulation was caused by losses of chloroplastic H_2O_2 scanvengers (Figure 3.7 D). Consistent with the hypothesis, *tAPX* promoter activity was induced in *2cpb*, *2cpa2cpb* (Figure 3.7 C). Noticeably, *tAPX* promoter activity was induced in *2cpa*, in which H_2O_2 level was not changed. As mutation of *2CPA* disturb the chloroplast H_2O_2 removing capacity, the minor change of which is not visible by measuring the overall H_2O_2 level from leaf extract, it is reasonable to maintain that the increased *tAPX* promoter activity was due to moderate H_2O_2 level increase in *2cpa*. This showed that the *tAPX* expression indeed correlated the need for its antioxidant function, and the coordination is mediated by the chloroplastic H_2O_2.

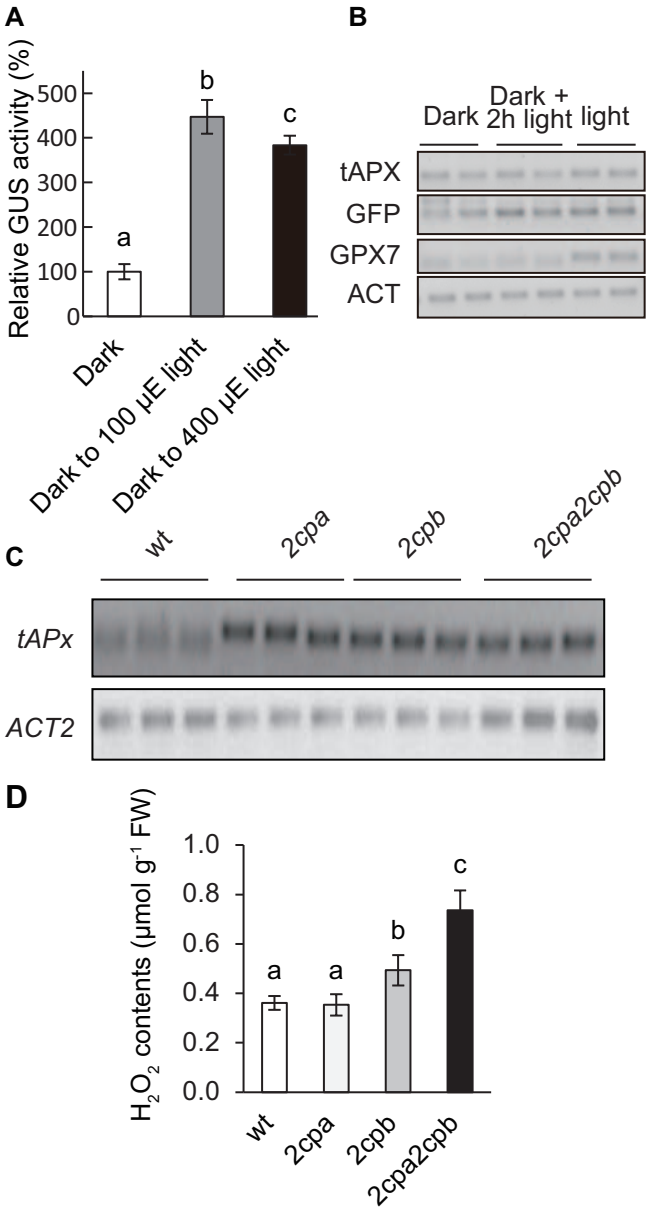

Figure 3.7 Effect of chloroplastic H_2O_2 on *tAPX* promoter activity. (A) 3 weeks old *pro*tAPX:EGFP-GUS seedlings were transferred to the dark for 24 hours, 100 or 400 µmol photons m^{-2} s^{-1} at the dark to light switch point and kept for 2 hours. GUS activity measurement followed. Data refer to the average of normalized 3 independent T3 transformation lines × biological replicates (±SE); different letters indicate pronounced difference, Student's t-test, n=10, $p \leq 0.05$. (B) RT-PCR was conducted in 3 weeks old *pro*tAPX:EGFP-GUS seedlings transferred to the dark, 2 hours 100 µmol photons m^{-2} s^{-1}, or plant constantly grow in 100 µmol photons m^{-2} s^{-1} light conditions. Each treatment was replicated twice with plants grown in different Petri dishes. (C) *tAPX* expression in chloroplast antioxidant deficient mutants. RT-PCR was conducted in 3 weeks old *2cpa, 2cpb*, and *2cpa2cpb* seedlings growing in normal conditions. Each treatment was replicated twice with plants grow in different petri dishes. (D) H_2O_2 contents in 3 week old *2cpa*, *2cpb*, and *2cpa2cpb* mutants. Data refer to the average of normalized 3 independent T3 transformation lines × biological replicates (±SE); different letters indicate pronounced difference, Student's t-test, $p \leq 0.05$, n=6.

3.3.5 Long term light stress

The plant transcriptome varies during the course of light stress (Vogel et al., 2014). Plant systematically switches its genes according to the requirement. With the stress persisting, a robust induction of certain genes is followed by the attenuation (Avraham and Yarden, 2011). This mechanism minimizes the damaging effect and potential energy waste. The chloroplast antioxidant system is thought to be acclimated well under 100 folds light stress (Oelze et al., 2012). Oxidative stress is relatively relieved at the later time point. A previous experiment demonstrated that the early light response of *tAPX* (Figure 3.7 A). The coming question is whether this induction can be reduced in the later course of light stress.

Transcription of *tAPX* promoter driven GFP was compared in plants subjected to 100 and 400 µmol photons m^{-2} s^{-1} for 4 hours and 36 hours after transferring from 24 hours dark adaption (Figure 3.8 A, B, and C). The result showed that short term (4 hours) exposure of the dark acclimated plants to light elevated the reporter gene expression, while long term exposure (36 hours) decreased the expression (Figure 3.8 D, E, and F). The control mechanisms of short and long light response are thus different. As previous experiment showed a promoting effect by chloroplastic H_2O_2 (Section 3.3.4), the up regulation by short period of light is triggered by the elevation of chloroplastic H_2O_2. Prolonged dark treatment decreased *tAPX* promoter activity (A and D in Figure 3.8). Most strikingly, 36 hours constant 100 µmol photons m^{-2} s^{-1} light decreased the reporter gene expression. *tAPX* is not a circadian regulated gene (Covington et al., 2008). The decrease of *tAPX* promoter activity in prolonged light is only due to light. A possible explanation is that the accumulation of a yet unknown factor under continuous light controls the promoter activity of *tAPX* in order to avoid the potential harm of unfettered prolongation of the elevated activity. Thus light signal forms an antagonistic regulation in short and long term for *tAPX* expression.

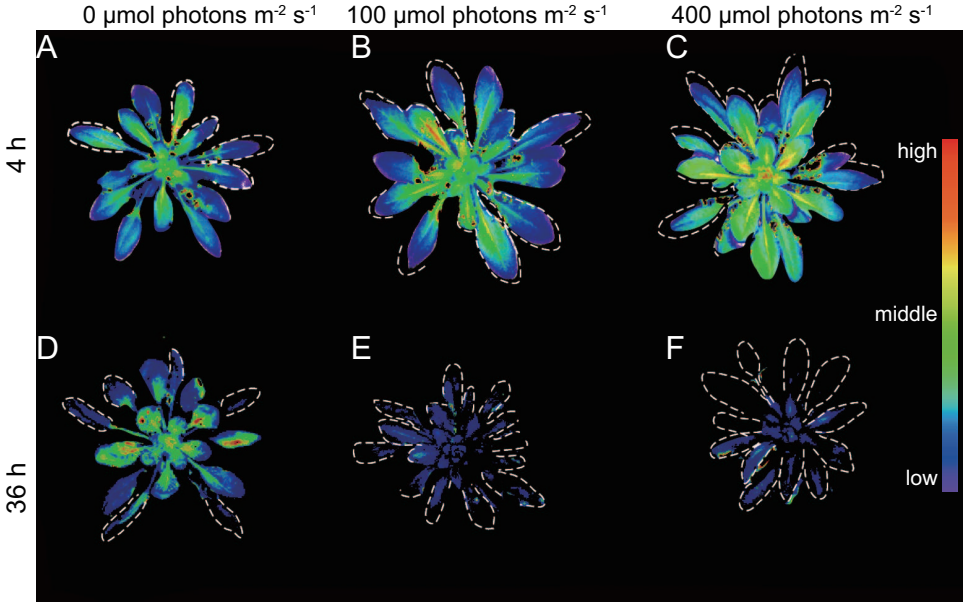

Figure 3.8 Effect of long and short light on *tAPX* promoter activity. The 5 weeks old *pro*tAPX:EGFP-GUS plants were grown under normal growth condition, and put under dark for adaption for 24 hours, and then transfer to 0, 100, and 400 µmol photons m^{-2} s^{-1} light for 4 or 36 hours. Fluoresce of EGFP was imaged by NightSHADE.

3.3.6 Regulation of *tAPX* promoter by different quality of light

As seen from the *in silico* analysis, the *tAPX* transcript levels are regulated by different quality of light. The following experiment investigates the regulation of *tAPX* promoter by

light is at transcription level. *tAPX* promoter driven expression of reporter gene can be gradually lost in dark conditions (Figure 3.9 A). Noticeably, the GUS activity in hypocotyl remains detectable on the 6th day in the dark, while that in leaves completely is lost on the 4th day. The lost expression can be rescued by 8 hours of additional white light, or certain mono-chromatic light, blue or far-red light (Figure 3.9 B). However, the expression cannot be recovered by exposing the dark adapted plants to red light. This result indicates that the promoter regulation of tAPX is subjected to specific light receptor mediated signaling, although the nature of mediator needs to be elucidated.

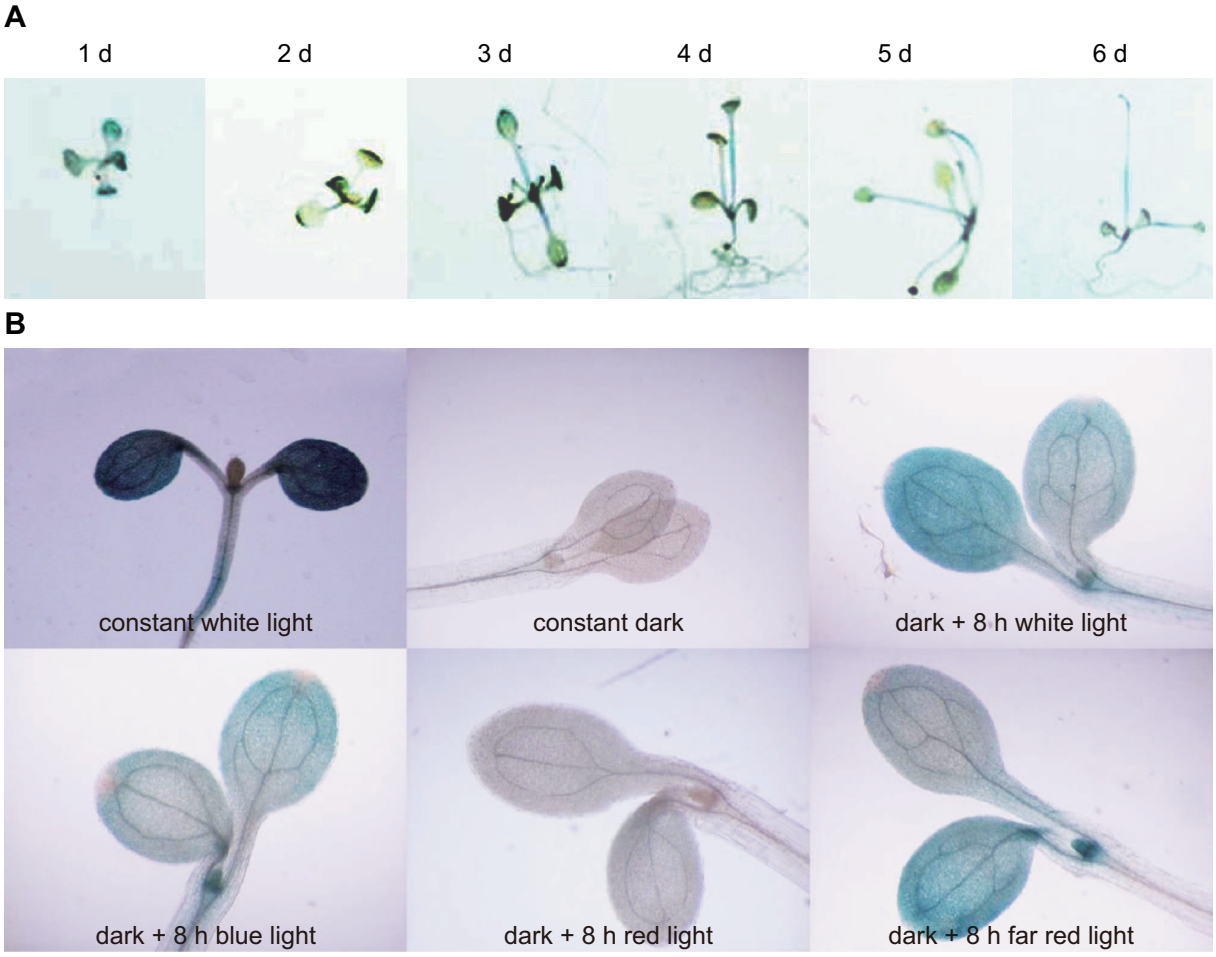

Figure 3.9 Light quality regulation of *tAPX* promoter. (A) Dark induced reduction of *tAPX* promoter activity. *pro*tAPX:EGFP-GUS were grown on 1/2 MS media for 7 days. GUS staining were performed indicated days after put into dark conditions. (B) Effect of light spectrum on *tAPX* promoter activity. *pro*tAPX:EGFP-GUS were grown on 1/2 MS media for 7d constant light , 7d dark , 7d dark followed by 50 µmol photons m^{-2} s^{-1} white light , blue light , red light, or far red light. GUS staining of seedlings was followed after light treatment.

3.4 Transcriptional regulation of *tAPX* by its *cis*-motifs and corresponding transcription factors

3.4.1 Localization of the H_2O_2 regulated element of *tAPX* promoter and its corresponding transcription factor prediction

In order to localize the chloroplastic H_2O_2 regulated motif within the *tAPX* promoter (-1571 bp to -1 bp), the promoter was truncated into 3 fragments with different length, -741 bp to -1 bp, -528 bp to -1 bp, -211 bp to -1 bp (Figure 3.10 A). The truncated promoters were fused to the reporter gene EGFP:GUS like the full length promoter construct and transformed to Arabidopsis following the same procedure for *pro*tAPX:EGFP-GUS. GUS staining was performed in the T2 generation. Figure 3.10 B showed that the promoters shorter than -741 bp to -1 bp were not able to promote *tAPX* expression in leaf blade tissues. Weak expression at the petiole end was seen, where mechanical injures was inevitably caused by cutting the leaves from the plants. The promoter motif regulating th wounding response of *tAPX* gene is localized within the -211 bp to -1 bp, as the shortest construct had the similar wounding dependent GUS staining pattern as longer ones (Figure 3.10 B). In the contrast, -1571 bp to -1 bp drove the reporter gene expression in the mesophyll cells. Thus, the motif causing a relatively high expression of GUS is localized within the -1571 bp to -741 bp region in the *tAPX* promoter. Comparing with wounding caused expression in other tissues, the GUS expression in leaf blade accounted nearly all the activity measured by quantitative GUS assays, by whose results chloroplastic H_2O_2 regulation of *tAPX* promoter was shown. Therefore, it is reasonable to assert the chloroplastic H_2O_2 regulating element of *tAPX* promoter is within 1571 bp to -1 bp.

Athamap (http://www.athamap.de/) was used to predict the transcription factor binding motif within *tAPX* promoter (Bulow et al., 2010). The putative transcription factors and their binding motifs which present in -1571 to -741 bp but not within -741 to -1 bp is showed in Figure 3.10 C (the comparison of -1571 bp to -1 bp and -741 bp to -1 bp, see supplementary **Supplementary Data 5** A, and B). The putative transcription factors are LEC2 (Leafy Cotyledon 2), SPL3 (Squamosa Promoter binding protein-Like), SPL8, ARR2 (Type-A Response Regulator 2), and ARR10. There were few reports on regulation of chloroplast antioxidant system by those transcription factors. LEC2 is a seed-specific transcription factor important for embryo development. Its null mutant is lethal. It binds to RY-motif (CATGCA), and regulates seed embryogenesis (Braybrook et al., 2006). It is unlikely that LEC2 regulates the expression of genes for chloroplast antioxidant enzymes in leaves. SPL transcription

factors (SPL3 and SPL8) are switches for juvenile-adult vegetative-flowering growth, and its expression increases with development. Both SPL3 and SPL8 bind to GTAC core motif within gene promoter, while SPL3 has a preference for sequences with the 5'-end of the GTAC motif (Birkenbihl et al., 2005). The putative binding of SPLs to the *tAPX* promoter may negatively regulate the gene, and thus cause an ageing dependent decrease expression of *tAPX* (Panchuk et al., 2005). Moreover, the SPLs are regulated by miR156 (Wang et al., 2009). Overexpressing miR156a causes an ectopic expression of *tAPX* in floral apex (Schwab et al., 2005) (GEO dataset: GSE2079). ARR2 and ARR10 are important for the two-component signaling of cytokinin response (Ishida et al., 2008). Both ARR2 and ARR10 bind to the AGATT motif within the promoter (Sakai et al., 2000; Hosoda et al., 2002). Constitutive activation of an overexpressed ARR2 represses *tAPX* expression (Hass et al., 2004). In summary, it is tempting to test the effect of SPL3, SPL8, ARR2, and ARR10 on the expression of *tAPX*.

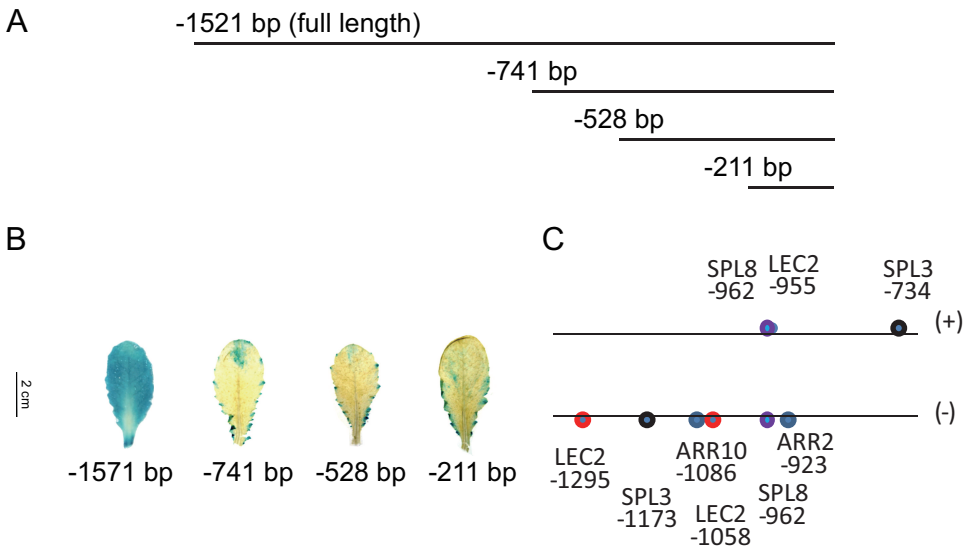

Figure 3.10 Localization of critical regulating element of *tAPX* promoter. (A) The truncation of *tAPX* promoter. Upstream promoters of *tAPX* with indicated length (start coden=+1), were cloned to generate reporter gene lines. (B) GUS staining of lines carrying indicated length *tAPX* promoters fused to EGFP:GUS. (C) Putative predicted transcription binding site in the critical region, as shown in (B).

3.4.2 The verification of regulation of *tAPX* promoter activity by ARR2, ARR10, SPL3, SPL8

3.4.2.1 ARR2

ARR2 (At4g16110.1) is a pollen-specific transcription factor (Lohrmann et al., 2001). Therefore it is unlikely that ARR2 regulates the *tAPX* expression in plant leaves. Hass et al. (2004) observed a dramatic repression of tAPX in lines over-expressing D80E ARR2 (constitutive active form) compared with lines overexpressing of ARR2 (inactive form). This showed the potential of artificial constitutive active form of ARR2 regulating *tAPX* promoter negatively, possibly by direct binding. However, the lack of this regulator in leaves make *tAPX* expression free form the control of ARR2. The transcript abundance was checked of *tAPX* in a homozygous T-DNA line of ARR2 (SALK_043107). The result demonstrates no significant change of *tAPX* expression in *arr2*, which is consistent with the microarray analysis performed by Hass et al. (2004). In summary, the *tAPX* promoter is not regulated by ARR2.

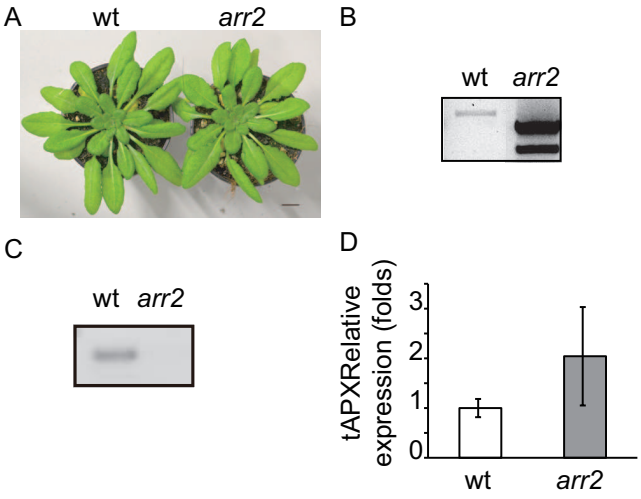

Figure 3.11 The expression of *tAPX* in *arr2*

(A) Phenotype of *arr2* compared with wild type. Plants are 4 weeks old. (B) Genotyping PCR confirmed that the line used is a homozygous plant of T-DNA insertion at the *arr2* gene locus. Multiple PCR with three primer sets clearly distinguish wild-type (single larger band), homozygous (single smaller band) and homozygous (both bands) DNA. The lower bind in *arr2* might due to the unspecific binding. (C) RT-PCR result indicated that the *ARR2* expression was knocked down. (D) The expression of *tAPX* in the *arr2* plant is comparable with wild type plant. Plants used were 4 weeks old. Student's t-test indicated there is no significant difference, n=6.

3.4.2.2 ARR10

ARR10 (At4g31920.1) is another type-B ARR predicted to bind the -1571 bp to -741 bp region of *tAPX* promoter (binding to -1086 bp of *tAPX* promoter, see Figure 3.10). The homozygous *arr10* (SALK_025664) plant does not express *ARR10* gene (Figure 3.12 B and

C). The expression of the *tAPX* in the knock out line, however, did not change. This indicates that *tAPX* promoter is not regulated by ARR10.

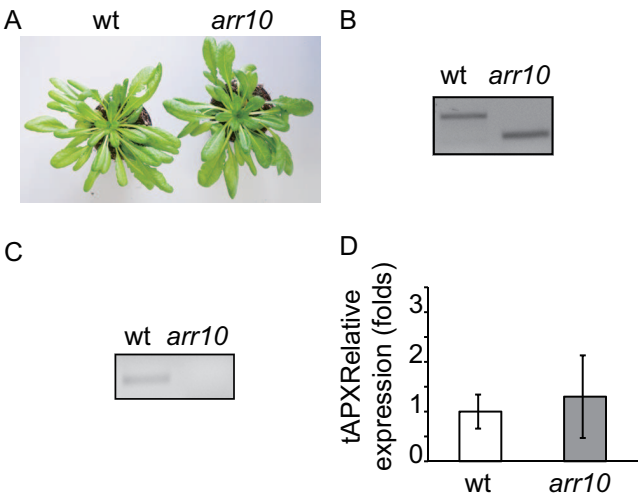

Figure 3.12 The expression of *tAPX* in *arr10*

(A) Phenotype of *arr10* compared with wild type. Plants are 4 weeks old. (B) Genotyping PCR confirmed that the line used is a homozyogous plant of T-DNA insertion at the *ARR10* locus. (C) RT-PCR result indicated that the *ARR10* gene expression was knocked down. (D) The expression of *tAPX* in the *arr10* plant is comparable with wild type plant. Plants used were 5 weeks old. Student's t-test indicated there is no significant difference, n=6.

3.4.2.3 SPL3

SPL3 (AT2G33810.1) was predicted to bind to the -1571 bp to -741 bp of *tAPX* promoter. The biological relevance of this binding is to be investigated here. Both *spl3-1* and *spl3-2* are lines with T-DNA in the -1000 bp promoter region. *spl3-2* has lower transcript level of *SPL3* than *spl3-1* (Figure 3.13 C). Neither of the mutations caused obvious effects on the plants phenotype at its vegetative growth stage (Figure 3.13 A). However, the expression of *tAPX* was dramatically promoted in this line. Thus *tAPX* transcription was negatively regulated by SPL3, through a putative binding. A recent study suggested that the miR156-SPL modulates memorial response on the recurrence of heat shock, and integrate the stress responses with development (Stief et al., 2014). The repression effect seen in our result indicates that SPL regulates the chloroplast antioxidant system. Whether SPL is regulating other antioxidant enzymes need to be further investigated.

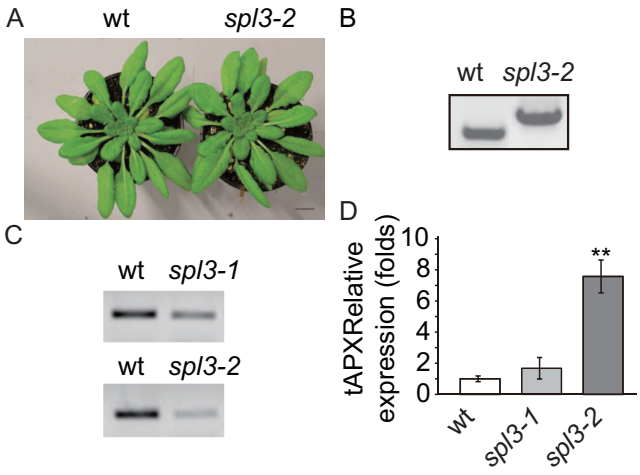

Figure 3.13 The expression of *tAPX* in *spl3*

(A) Phenotype of *spl3* compared with wild type. Plants are 4 weeks old. Since *spl3-1* (SALK_035860) and *spl3-2* (SALK_035917) and wild type share the same morphology, only *spl3-2* and wild type were displayed here. (B) Genotyping PCR confirmed that the line used is a homozygous plant of T-DNA insertion at the *SPL3* gene locus (*spl3-1* is the same with *spl3-2*). (C) RT-PCR result indicated that the *spl3-2* gene expression was knocked down to a higher extend than *spl3-1*. (D) The expression of *tAPX* in the *spl3-1* and *spl3-2* plant is comparable with wild type plant. Plants used were 4 weeks old. ** indicates a significant difference Student's t-test, $p<0.05$, n=6.

1.1.1.1. SPL8

In order to test whether SPL8 (At1g02065.1) regulates transcription of *tAPX*, a homozygous T-DNA insertion line (SAIL_816_E01) was used (Figure 3.14 B). In the mutant the expression of *SPL8* was knocked out (Figure 3.14 C). Plants grown on soil showed no obvious phenotype compared to wild type (Figure 3.14 A). The expression of *tAPX* however was elevated by over 6 folds. This demonstrates a negative regulation of *tAPX* promoter by SPL8. SPL8 binds to a palindromic sequence at position of -962 bp of tAPX promoter (Figure 3.10). *In vitro* assay need to be done in the future to confirm the binding. The negative regulation can be explained in a way that the binding of SPL8 inhibits binding of a positive regulator. SPL8 is a developmental switch regulator. The repression effect by SPL8 underlies the decrease of *tAPX* expression with development.

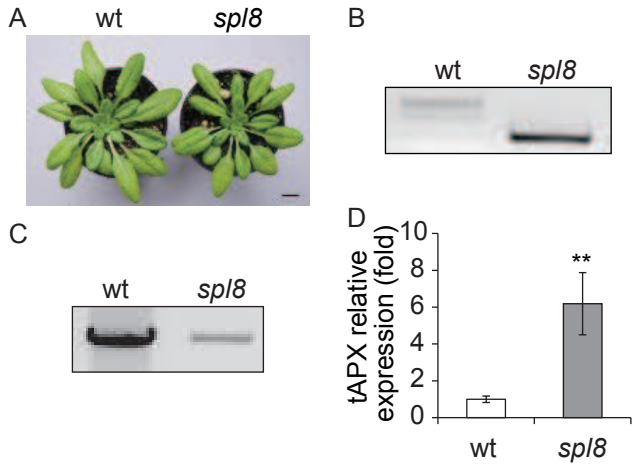

Figure 3.14 The expression of *tAPX* in *spl8*

(A) Phenotype of *spl8* compared with wild type. Plants are 4 weeks old. (B) Genotyping PCR confirmed that the line used is a homozygous plant of T-DNA insertion at the *SPL8* locus. (C) RT-PCR result indicated that the *SPL8* gene expression was knocked down. (D) The expression of *tAPX* in the *spl8* plant is compared with wild type plant. Plants used were 4 weeks old. ** indicates a significant difference, Student's t-test, $p<0.05$, n=6.

3.5 Transcriptional regulation of *sAPX* promoter

As in section 3.2.1, the *sAPX* promoter activity is much lower in mesophyll cells compared with that of *tAPX* promoter. The majority of *sAPX* promoter activity was observed in the vasculature. Thus, more attention was given to the regulation of *sAPX* in vasculature. The transcriptional regulation of *sAPX* promoter in leaf blades is also of interest in this study, since sAPX is a protein function in chloroplasts which are mainly in mesophyll cells. In order to visualize the low expression of *sAPX* promoter driven GFP expression using a Nightshade fluorimeter, the exposure time has to be set to very long (3 times longer than that for *tAPX* promoter construct).

3.5.1 Cold regulation of *sAPX*

Juszczak et al. (2012) described the accession dependent regulation of *sAPX* gene expression by cold, *i.e.* response of accession used to extreme habitats, Kas-1 from alpine and Cvi-0 from a warm and humid habitat, were different in ecotypes from a more moderate habitat. The mRNA levels of *sAPX* in the cold experiments are elevated compared to 20 ºC grown accessions originated for the moderate climate. To verify whether the transcript abundance elevation is due to transcriptional control in Col-0, *pro*sAPX:EGFP-GUS was tested for cold responsiveness. As shown in Figure 3.15 A, the *sAPX* promoter was dramatically elevated upon cold treatment. The elevated expression was more obvious in young leaves, where chloroplasts develop and are more sensitive to oxidative stress (Sakamoto et al., 2013). Surprisingly, the elevated expression showed dot-pattern on the leaf surface (Figure 3.15 A). It is likely that the dots are in the leaf trichomes. Further study by florescence microscopy confirmed this assumption (Figure 3.15 B). The cold induced promotion of *sAPX* transcription

is contrasting to the repression of *tAPX* promoter as seen in section 3.3.3. This demonstrates that the preference of two isoforms of chloroplastic APX varies with the conditions.

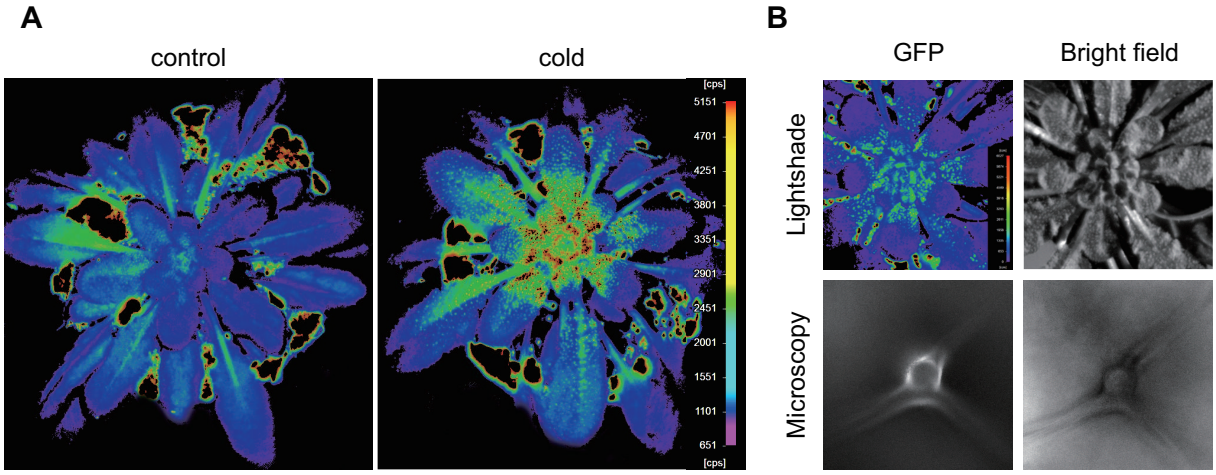

Figure 3.15 Effect of cold on *sAPX* promoter activity. The 5 weeks old *pro*sAPX:EGFP-GUS plants were grown under normal growth condition, and put under 20 °C (control) or 4 °C (cold) conditions for 24 hours. Fluorescence of EGFP was imaged by NightSHADE. (B) Cold induction of *sAPX* promoter activity in plant leaf trichome. Fluoresce pictures were taken under NightSHADE fluorimeter and confocal microscopy. Bright field pictures were taken within the same scopes.

3.5.2 *sAPX* promoter activity increases under drought condition

Drought stress makes plants to shut their stomata, which are the entrance sites of CO_2 (Flexas and Medrano, 2002). A decreased CO_2 assimilation rate causes accumulation of ROS in chloroplasts (Lawlor and Cornic, 2002). *tAPX* transcription was shown to be down regulated by drought (section 3.3.3). To test whether *sAPX* promoter activity is regulated by drought, the response of the *sAPX* promoter was tested by *pro*sAPX:EGFP-GUS. In comparison with the low expression of *sAPX* driven expression reporter gene in the non-treated plants, the expression was enhanced by drought stress (**Error! Reference source not found.**). This is consistent with the previous observation that the preference of two isoforms of chloroplastic APX varies with the conditions. The elevation of *sAPX* expression could balance the decrease of tAPX. The reason for what plants prefer sAPX under such conditions is yet not clear. However, the overall ability of maintaining reduced/oxidized ascorbate converting is important for plant drought resistance (Arase et al.; Niu et al., 2013).

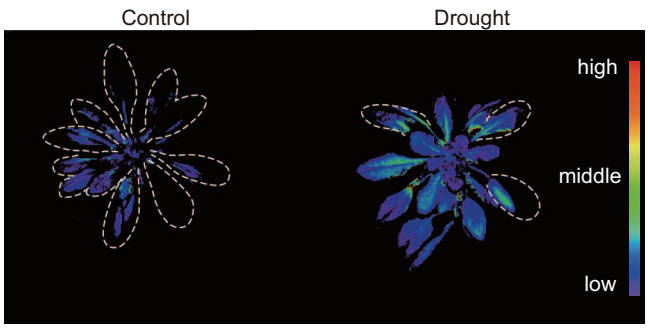

Figure 3.16 Effect of drought on *sAPX* promoter activity. The 5 weeks old *pro*sAPX:EGFP-GUS plants were grown under normal growth condition, and put under control (normal watering regime) or drought condition (3 days without water, and leaves turn red). Fluorescence of EGFP was imaged by Nightshade fluorimeter.

3.5.3 Effect of chloroplast function on *sAPX* promoter

The GUS staining pattern in seedlings demonstrated that *sAPX* promoter activity is stronger in veins than in other parts of the mesophyll cells (section 3.2.2), where the weak expression can be detected by using the fluorimeter system. As leaf vasculature is not the major photosynthesis site, it is of interest whether the chloroplast functional integrity has a role in regulating *sAPX* promoter. The proper function of chloroplasts is required for transcription of many nucleus encoded chloroplast localized proteins (Ruckle et al., 2007). To test whether the loss of function of chloroplasts has an effect on expression, lincomycin and NF were used. Lincomycin inhibits the chloroplast protein biosynthesis (Ellis, 1975). NF inactives phytoene desaturase (a key enzyme for carotenoid biosynthesis) and thus causes photo-damage for plants in the light (Breitenbach et al., 2001). The results here indicates a malfunction of chloroplast, which decreases the expression of *sAPX* irrespective of whether it was caused by linocmycin or NF (Figure 3.17 A and B). Chloroplast function controls *sAPX* expression. The reduction, however, is limited in the mesophyll cells, while the leaf vein retains its GFP expression after being treated with lincomycin and NF (Figure 3.17 A). Apparently, these chloroplast affecting chemicals influenced *sAPX* expression less effectively in tissues with less chloroplasts. The expressions of *sAPX* in vein and mesophyll cells are controlled by different signals.

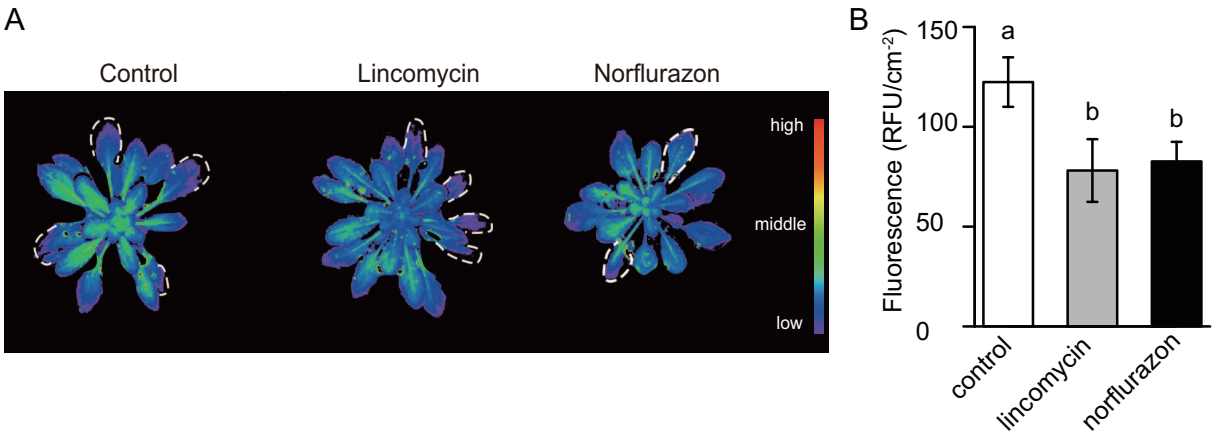

Figure 3.17 Effect of licomycin and norflurazon on *sAPX* promoter activity. 5 weeks old *pro*sAPX:EGFP-GUS plants were grown under normal growth condition. 25 µM Lin, or NF were sprayed and then infiltrated by vacuum. Water was used as control. Fluorescence of EGFP was imaged by NightSHADE fluorimeter. (A) Representative picture were shown here as control. (B) Fluorescence was quantified and normalized to relative fluorescence units in leaf area measured (cm^{-2}). Different letters above bars indicate a significant difference Student's t-test, $p<0.05$, n=30.

3.5.4 Regulation of *sAPX* promoter by light intensity: repression and activation

Regulation of *sAPX* promoter by light: repression and activation at the same time

As evidence in the above section illustrates that a signal from chloroplast impacts on *sAPX* expression in the leaf blade. It is tentative to deduce whether light regulates *sAPX* expression. Light alters the chloroplast physiological state, such as initiating metabolisms including photosynthesis, varying ROS levels, even changing the chloroplast structure (Lichtenthaler et al., 1981). To verify the impact of light on *sAPX*, varies of intensity of illumination (0, 100, and 400 µmol photons m^{-2} s^{-1}) on *pro*sAPX:EGFP-GUS plants for 4 hours were used. The expression of the reporter gene, EGFP, showed the highest expression in dark treated plants. The 400 µmol photons m^{-2} s^{-1} treated plants had slightly higher expression of EGFP than 100 µmol photons m^{-2} s^{-1} treated (Figure 3.18). Moreover, the elevated expression in dark treated plants were mostly seen in leaf midrib in both young and old leaves, while the 400 µmol photons m^{-2} s^{-1} light up-regulated EGFP expression was seen in younger leaf blades.

Similar set-up was used to detect the long termed (36 hours) light effect. The response of *sAPX* promoter to the same light intensity for 36 hours after dark adaption was studied. Strong difference of GFP expression in 3 light conditions exposed plants was seen compared with that of short long light treatment (Figure 3.18 lower row). In comparison with 36 hours dark treated plants, plants exposed to 100 µmol photons m^{-2} $s^{-1}$and 400 µmol photons m^{-2} s^{-1} light showed much lower *sAPX* driven reporter expression (Figure 3.18 lower row). To visualize

the GFP signal, the heat map scale had to be so minimized that the expression in dark treated plant went above threshold of the same scale (Figure 3.18 lower row: dark area within leaf boundary in left most picture indicates spillover of GFP signal above the heat map scale). However, there is little difference between the long term of 100 and 400 µmol photons m^{-2} s^{-1}. In the leaf blade, especially in young leaves, the expression of *sAPX* is lower than that in dark plant. Therefore, the promoter activity of *sAPX* in leaf blade went up when transferred from dark to light condition (short term), and dropped when light exposure was prolonged.

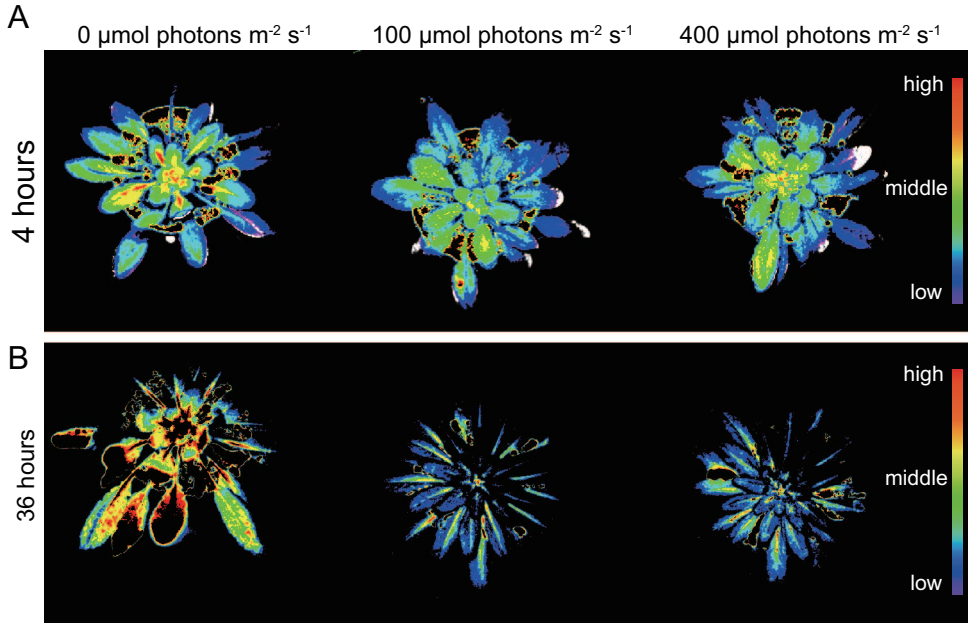

Figure 3.18 Effect of light on *sAPX* promoter activity. 5 weeks old *pro*sAPX:EGFP-GUS plants were grown under normal growth condition. 4 hours (upper row) or 36 hours (lower row) exposure to 0, 100, and 400 µmol photons m^{-2} s^{-1} light was treated after plants were dark adapted for 24 hours. Fluorescence of EGFP was imaged using NightSHADE fluorimeter. Representative picture were shown here as control.

3.5.5 Effect of light spetrum on *sAPX* promoter

Light quality regulation of the *sAPX* promoter activity was analyzed by illumination with blue, red, and far-red light. During up to 3 days dark treatment, the chloroplasts are transformed to etioplasts, a form of chloroplast not capable of photosynthesis (Solymosi and Schoefs, 2010). However, some photoreceptors remain functional in plant cell (Kuno et al., 2000; Deng et al., 2014). In this experiment, same light intensity but different spectrum of light was used to treat the plants, with dark environment as a control (Figure 3.19 A and B). Expression of *sAPX* promoter driven reporter level was very low in every treatment, yet the induction effect of 100 µmol photons m^{-2} s^{-1} blue light is still visible. The dark induction or light repression of *sAPX* promoter driven GFP expression (section 3.5.4) was replicated by 100 µmol photons m^{-2} s^{-1} blue light. Blue light exposure made the expression in the vein

disappear (arrows in Figure 3.19). Blue light also caused a dot-pattern induction of GFP expression in the young leaves. The induction of reporter gene patterns as dots in young rosette leaves is like that in drought and cold induction (section 3.3.5 and 3.3.5). Perhaps, the induction of *sAPX* promoter activity in young leaves may be stimulated by multiply factors. The red light or the far-red light could neither suppress nor promote the activity of the *sAPX* promoter. Blue light related receptors are involved in the *sAPX* transcription regulation.

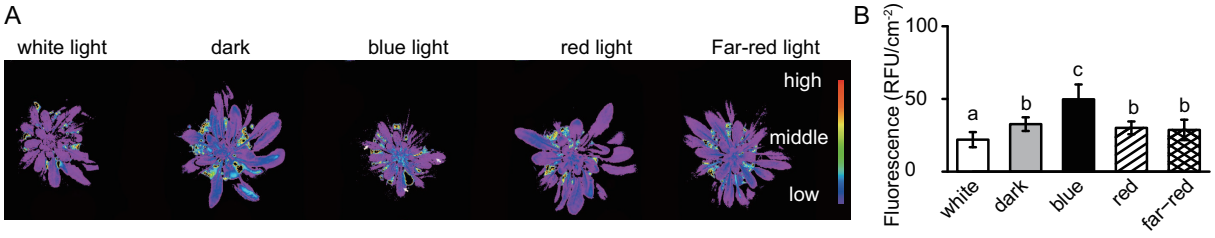

Figure 3.19 Effect of light spectrum on *sAPX* promoter activity. 5 weeks old *pro*sAPX:EGFP-GUS plants were grown under normal growth condition. Plants were subjected to 24 hours of 100 µmol photons m^{-2} s^{-1} white light, dark, 100 µmol photons m^{-2} s^{-1} red light, blue light, and far-red. Fluoresce of EGFP was imaged using NightSHADE fluorimeter. (A) Representative picture were shown here as control. (B) Fluorescence was quantified and normalized to relative fluorescence units in leaf area measured (cm^{-2}). Different letters above bars indicate a significant difference Student's t-test, $p<0.05$, n=30.

3.5.6 Effect of photosynthetic electron transport on *sAPX* promoter activity

The next experiment was to show whether the photosynthetic electron transport chain regulation on *sAPX* is same as that of the *tAPX* promoter which is regulated by redox/H_2O_2 downstream of PQ pool (section 3.3). To test whether *sAPX* is regulated in the same way, the same herbicides as in section 3.3 were used. Like the *tAPX* promoter, the *sAPX* promoter does not respond to the redox changes caused by DCMU and DBMIB (Figure 3.20 A and B). A slight decrease of *sAPX* promoter activity was seen in the young leaves caused by DCMU and DBMIB. Young leaves are more responsive to stresses. However, *sAPX* promoter driven GFP expression was dramatically increased in MV treated plants. MV treated plant causes H_2O_2 accumulation inside the chloroplast (Hatzios et al., 1980). The results here demonstrated that *sAPX* and *tAPX* are regulated in the same "direction" (up or down regulation) and perhaps by the same signal in terms of chloroplast PET. Again the result here demonstrated that the expression of *sAPX* in leaf vasculature is not altered by those PET blocking agents, confirmed the assertion that chloroplast signal does not regulate the *sAPX* expression in vasculature.

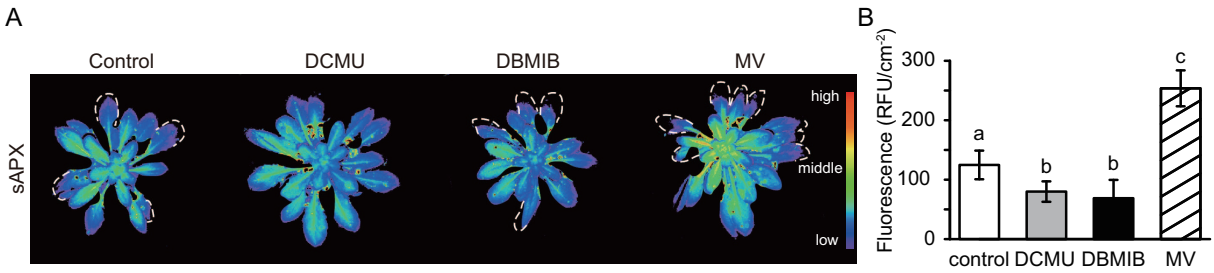

Figure 3.20 Effect of photosynthetic electron transport on *sAPX* promoter activity. 5 weeks old *pro*sAPX:EGFP-GUS plants were grown under normal growth condition. 25 µM DCMU, DBMIB, or MV were sprayed and vacuumed. Water was used as control. Fluoresce of EGFP was imaged using NightSHADE fluorimeter. (A) Representative picture were shown here as control. (B) Fluorescence was quantified and normalized to relative fluorescence units in leaf area measured (cm^{-2}). Different letters above bars indicate a significant difference Student's t-test, $p<0.05$, n=30.

3.5.7 *sAPX* regulated by phytohormones

To investigate possible hormone cues that can integrate the sugar sensing signal, the response of the *sAPX* promoter to hormones (IAA, BAP, ABA, JA, and SA) were tested. A slight increase of the *sAPX* expression was seen in JA treated plant. Given the *tAPX* expression does not change much under the JA treatment; the moderate but significant increase of ascorbate peroxidase activity as seen in a previous study could be attributing to the slight increase of *sAPX* expression (Sasaki-Sekimoto et al., 2005). In the following experiment, the *pro*sAPX:EGFP-GUS were sprayed with different hormones (Figure 3.21 A and B). The results demonstrated that there was no dramatic effect of IAA, BAP, ABA, JA, and SA on *sAPX* promoter, while cytokinin (1 mM BAP) elevated the promoter activity of *sAPX.* Transport of BAP is carried out in the plant xylem system. Its high concentration in the vasculature coincides with the promotional effect.

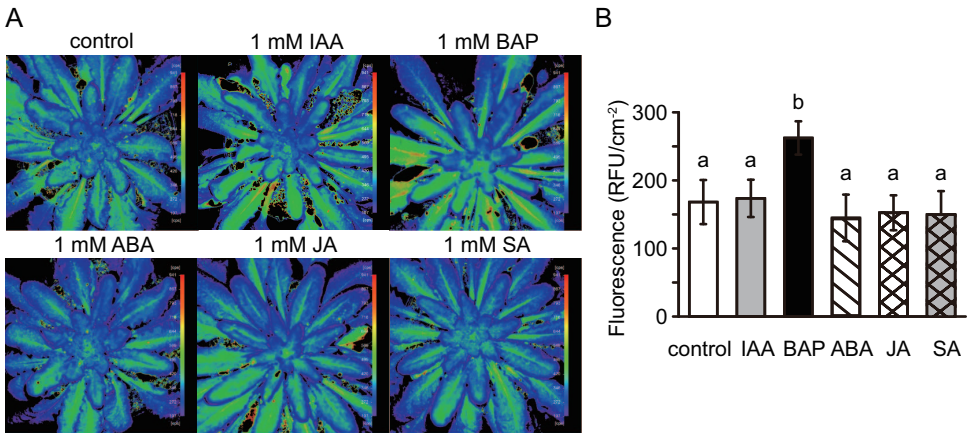

Figure 3.21 Effect of auxin, cytokinin, abscisic acid, jasmonic acid, and salicylic acid on *sAPX* promoter activity. 5 weeks old *pro*sAPX:EGFP-GUS plants were grown under normal growth condition. 1 mM auxin (IAA), cytokinin (BAP), abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA) were sprayed and vacuumed. Water was used as control. Fluoresce of EGFP was imaged by NightSHADE. (A) Representative picture were shown here as control. (B) Fluorescence was quantified and normalized to relative fluorescence units in leaf area measured (cm^{-2}). Different letters above bars indicate a significant difference Student's t-test, $p<0.05$, n=30.

3.5.8 Transcriptional regulation of *sAPX* promoter by its *cis*-motifs and corresponding transcription factors

Similar with in section 3.4.1, truncated promoters with different length, -1989 to -40 bp (un-truncated full length promoter), -1281 to -40 bp, -654 to -40 bp, -378 to -40 bp, -222 to -40 bp (Figure 3.22A), were fused to the reporter gene EGFP:GUS. Arabidopsis was transformed with these constructs in order to localize the regulating motif within the *sAPX* promoter. Overall, the expression of *sAPX* promoter driven expression of GUS was weaker than for *tAPX* promoter fragments. Expression of GUS was more obvious in veins than in the leaf blades. Promoter fragments shorter than -654 bp were not able to drive GUS expression in response to mechanical wounding (Figure 3.22 B). Promoters shorter than this length could drive the reporter expression in plant vasculature. However, the regulatory motif, as least to wounding, is missing within this region.

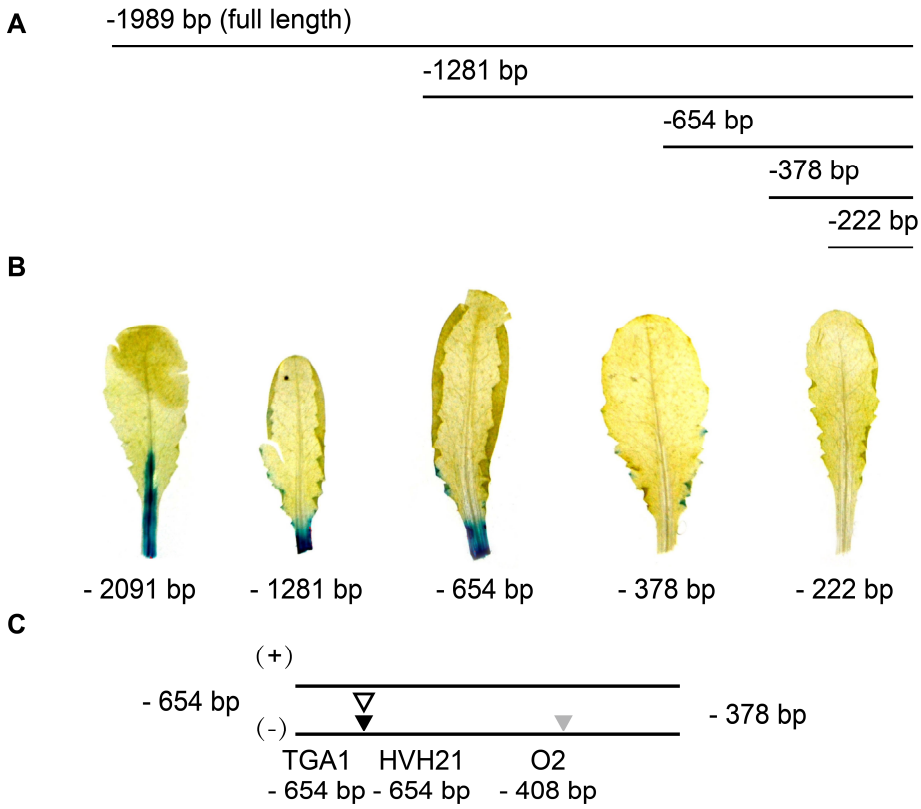

Figure 3.22 Localization of critical regulating element of *sAPX* promoter. (A) The truncation of sAPX promoter. Upstream promoters of *sAPX* with indicated length (start codon=+1), were cloned to generate reporter gene lines. (B) GUS staining of lines carrying indicated length *sAPX* promoters fused to EGFP:GUS. (C) Putative predicted transcription binding site in the critical region, as shown in (B).

Athamap (http://www.athamap.de/) was applied to predict the transcription factor binding motif within *sAPX* promoter and regulating *sAPX* expression (Bulow et al., 2010). 3 transcription factors were predicted to bind to the region of -654 bp to -378 bp, but not to promoters shorter than -378 bp. Thus the 3 transcription factors (TGA1, HVH21, and O2) are regarded as important for *sAPX* transcription regulation. TGA is a transcription factor binds to the 5'-TGACGTGG-3', which is similar to the G box (5'-ACGT-3') (Schindler et al., 1992). The binding of the TGA1 transcription factors to TGA motif is regulated by pathogen-attack and salicylic acid in a redox controlling manner (Lindermayr et al., 2010). S-nitrosylated and S-glutathionylated of cysteine residues within TGA1 in response to cellular redox state can modify the DNA binding activity of TGA1 (Lindermayr et al., 2010). HVH21 site (TGAC consensus sequence) binds home domain proteins of the knotted class 1 (Krusell et al., 1997). This motif also has the potential to bind bHLH (basic Helix-Loop-Helix) transcription factors, because the TGAC motif overlaps with the E-box (CANNTG) (Krusell et al., 1997; Wang et al., 2010). Whether the light regulation behavior of *sAPX* is attributed to the presence of this motif needs further investigation. As the TGA1, the HVH21 motif reposes to nitrate (Wang et

al., 2010). The O2 motif binds to a basic domain/leucine zipper (bZIP) OHP1 (O2 Heterodimerizing Protein 1) (Wang et al., 2010). This gene regulation by binding OHP1 to the O2 motif is important for plant seed development and not critical for vegetative growth (Gavazzi et al., 2007).

The significance of these predicted motifs needs further confirmation. The prediction by Athamap does not exclude other possible motifs. Actually, a NAC family transcription factor has been shown to bind and thus negatively regulate *sAPX* (Klein et al., 2012). The truncation experiment here demonstrates the presence of a positive regulating transcription factor binding site with -654 bp and -378 bp. However, previous attempts using Y1H to find the corresponding transcription factor had failed because of highly leaky expression (Klein et al., 2012).

4 Discussion

4.1 Tissue specificity of *sAPX* and *tAPX* promoter activity

As emphasized by Asada (1999), the identification of the control mechanism of the biosynthetic ratio of the two chloroplastic APX isoforms should help to elucidate their respective functions. Although lower expression of *sAPX* mRNA levels than *tAPX* mRNA level was observed in several researches (Meneguzzo et al., 1998; Kiddle et al., 2003b; Davletova et al., 2005; Hebbelmann et al., 2012). Since the tissue specificity of *sAPX* and *tAPX* expression was not noticed before, the distribution regulation of *sAPX* and *tAPX* was lost in the mist.

The histochemical study (Figure 3.3) showed that *sAPX* promoter is mainly expressed in the vasculature, both in leaves and roots. Promoter truncation assays confirmed it. This promoter regulation pattern is consistent with shorter promoters (section 3.5.8). From these observations, a question raises immediately: what is sAPX protein function in vasculature cells? A possible answer is that sAPX serves as an antioxidant in vasculature chloroplasts. In Arabidopsis, leaf vascular bundle cells comprise approximately 15% of the chloroplast containing cells, the vascular bundle sheath (Kinsman and Pyke, 1998). Besides that, the sAPX protein is dually localized in mitochondria and choroplasts (Chew et al., 2003). The finding of vasculature specificity of *sAPX* promoter (section 3.3) is consistent with the concept that the mesophyll and vascular bundle have different antioxidant networks (Doulis et al., 1997; Majeran et al., 2005). Other reported vascular specific antioxidant enzymes are chloroplastic glutaredoxins (Cheng et al., 2006), peroxiredoxin II-E (Majeran et al., 2005), and Cu/Zn SOD Genes (Hu et al., 2010). Earlier research demonstrated that plant bundle tissues process higher APX activity than mesophyll (Doulis et al., 1997). This observation may be attributed to the vascular specific expression of a cytosolic isoform of APX, APX2, or/and other APXs (Fryer et al., 2003). A more recent research found that not only the cytosolic isoform but also a chloroplast stromal APX in maize has as over 2 folds protein level in vascular chloroplasts as that in mesophyll cells (Friso et al., 2010). Moreover, in tobacco non-photosynthetic cell sAPX protein is showed to be localized in plastid (Madhusudhan et al., 2003). Thus it is unlikely that sAPX protein is transported from the site where it is synthesized. Taking these together, sAPX protein has higher specificity in plant vasculature. Here, the histochemical study of *sAPX* promoter (section 3.3) demonstrated that the transcriptional regulation makes the vasculature specificity of *sAPX* promoter.

The tissue specificity of *sAPX* promoter was not noticed before. For instance, when reviewing the respective importance of sAPX ant tAPX, Asada (1999) arbitrarily assigned the protein amount ratio of sAPX to tAPX as 1:1 in the chloroplast. Since, the sAPX is primarily expressed in vascular tissue; tAPX protein amount in mesophyll cell must be higher than sAPX. Because few transcriptome experiments distinguished vasculature and the mesophyll cells, the data on *sAPX* mRNA level originated from microarrays are inconsistent with previous knowledge. It was surprising that the expression of chloroplast *APX* can be detected in non-photosynthetic issue (Madhusudhan et al., 2003; Hong and Kao, 2008; Lin and Pu, 2010). Of the four chloroplastic isoforms of ascorbate peroxidases (OsAPx5, OsAPx6, OsAPx7, OsAPx8) in rice, the expression and osmotic stress induction of OsAPx8 in root indicate that the enzyme might have function other than protecting functional chloroplasts (Hong and Kao, 2008). The chloroplast stromal isoform, but not the thylakoid isoform, had been detected in the non-photosynthetic tobacco BY-2 (Bright Yellow) cell (Madhusudhan et al., 2003). In spinach, stromal and thylakoid isoforms are encoded in one gene, whose alternative splicing generates one tAPX and three sAPX. The regulator of such splicing, involving a splicing regulatory element in its pre-mature RNA, distributes more stromal isoform in spinach roots (Yoshimura et al., 2002). This indicated the preference of sAPX in non-photosynthetic tissue is conserved in plant.

It was observed that the loss of tAPX have more severe impacts on plant photosynthesis than loss of sAPX (Kangasjärvi et al., 2008; Maruta et al., 2010), although both chloroplast isoforms shares similar enzymatic properties (section 1.2.2.2). This paradox can be explained by that the local concentration of tAPX on thylakoid is sufficient to remove H_2O_2 on thylakoid before it diffuses to stroma (Asada, 1999). Following this deduction, however, there will be no need to express sAPX in plant mesophyll cells wherever tAPX is present. The histochemical study (Figure 3.3) on *sAPX* and *tAPX* promoter activity provides another possible explanation, *i.e.* the tAPX is the only active chloroplast APX in plant mesophyll (The leaf expression of sAPX is restricted in vascular tissue under light condition). Previous experiments indeed suggested that tAPX is sufficient to keep H_2O_2 at safe concentration (Miyake et al., 1992; Miyake, 2010). tAPX is localized in the vicinity of PSI in thylakoid membranes (Miyake and Asada, 1992). The high local concentration of tAPX, high ascorbate level, and high tAPX efficiency keep the H_2O_2 to a safe concentration of 0.3-0.4 nM (Miyake, 2010). Actually, the upstream ROS scavenger, thylakoid attaching SOD catalyzing O_2^- converting to H_2O_2 also exists as a single copy in chloroplast (Ogawa et al., 1995; Asada, 1999). Moreover, cytosolic APX provides a backup for the H_2O_2 detoxification. The diffusion

property of H_2O_2 entitles that the defused H_2O_2 is detoxified by cytosolic APXs (Davletova et al., 2005; Zhang et al., 2013). The promoter specify of *sAPX* and *tAPX* promoter also explain why the loss of sAPX in Arabidopsis cause a phenotype in seedling leaf veins while loss of tAPX causes reduced photosynthesis activity under excess light (Kangasjärvi et al., 2008).

Another interesting phenomenon observed in this study is that *sAPX* is highly expressed in plant leaf blades subject to long term dark treatment (Section 3.5.4). The increase of *sAPX* expression under long term dark treatment is consistent with results from RNA blot experiment (Mano et al., 1997). This peculiar expression pattern also suggests a specific function of the sAPX protein. Under this condition, the chloroplasts are transformed to etioplasts, which do not have structured thylakoid (Philippar et al., 2007). Consistent with losing thylakoid, tAPX expression decreases to undetected level (section 3.3.6). Nevertheless, APX function is absolutely necessary for those immature chloroplasts. Because chlorophyll biosynthesis is block at the transformation from protochlorophyllide to chlorophyllide in prolonged dark treated plant, the etioplasts accumulate protochlorophyllide (Griffiths, 1978; Erdei et al., 2005). When prolonged dark treated plants are transferred to light condition, protochlorophyllide promotes ROS production (Erdei et al., 2005). Since this process is fast, plants can only reckon on pre-existing antioxidant enzymes for ROS removal. Result in Section 3.3.6 and 3.5.4 suggested that it is sAPX, but not tAPX, secures the etioplast-chloroplast transformation. Figure 3.18 also indicated that mesophyll cells have the ability of *sAPX* expression, but under a regulation that is distinct with *tAPX*.

4.2 Chloroplastic H_2O_2 regulates the *tAPX* transcription.

The experiments with photoelectron transport blocking agents showed that MV, as the only one of the used photosynthetic transport chain blockers, DCMU, DBMIB, and MV, induced the *tAPX* promoter (Figure 3.4). The sites of action of these electron transport inhibitors are described in (Figure 3.4 A). Under illumination in the presence of DCMU or DBMIB, the PQ pool is oxidized or reduced, respectively. *tAPX* down regulation under both conditions indicated that *tAPX* promoter regulation is independent of the redox state of PQ pool. The direct consequence of MV treatment is different from DCMU and DBMIB treatment in two aspects: (1) redox change of cytochrome *b6f* and natural electron acceptors of PSI (Alfonso, 2000); (2) the production of both O_2^- and H_2O_2 (Mano et al., 2001). There are two lines of evidence supporting the promoting effect on the *tAPX* promoter by MV is attributed to H_2O_2. First, a SOD knock out mutant accumulating O_2^- and incapable of converting O_2^- to H_2O_2 in chloroplasts has lower *tAPX* expression than wild type (Rizhsky et al., 2003). Second, higher

H_2O_2 production in knock down mutants of 2CPA or/and 2CPB induces the expression of *tAPX* (Figure 3.7). The chloroplastic H_2O_2 regulation of *tAPX* transcription suggests an economic way how plants strictly allocate message for regulating stress response genes according to their requirement. Suppose such a condition that the chloroplastic H_2O_2 is produced at a constant rate meanwhile the amount of tAPX in chloroplasts is not sufficient to remove H_2O_2. The accumulation of chloroplastic H_2O_2 promotes the expression of *tAPX*. As a consequence, the increased tAPX activity scavenges H_2O_2, and attenuates the signal controlling *tAPX* expression. This feedback regulation tightly coordinates the tAPX to the chloroplast requirement.

H_2O_2 may regulate the transcription activity directly by affecting the binging affinity of the transcription factors to their corresponding gene promoter (Shaikhali et al., 2008). The expression and the function similarity of tAPX and 2CPA endow a speculation that the *tAPX* gene transcription is regulated in the same way, *i.e.* H_2O_2 affects the binding of transcription factor to its promoter. However, data in this study showed that the exotic and endogenic increase of cytosolic H_2O_2 and chloroplastic H_2O_2 had a different impact on the *tAPX* promoter. Stimuli which trigger H_2O_2 in other cellular compartments cannot produce the same promoting effect as chloroplast H_2O_2 does (Figure 3.6). These observations are in agreement with previous results that the H_2O_2 in the cytosol does not promote the expression of *tAPX* (Vanderauwera et al., 2005). The comparison of transcription profiles of mutants accumulating H_2O_2 in chloroplast and peroxisome also suggested that the signal originated from chloroplastic H_2O_2 is distinct with those from H_2O_2 in other compartments (Sewelama et al., 2014). Therefore, the role of chloroplastic H_2O_2 in regulating tAPX promoter is indirect, *i.e.* to exert the controlling role, chloroplastic H_2O_2 must be transform to other molecules that traffic to nucleus and regulate transcription. The high chemical reactivity of H_2O_2 make transformation relatively easy (Bienert et al., 2006). Kinases, for instance, is likely the mediator for the chloroplast signal to nucleus. The transcriptional control of tAPX responding to environmental clues seems to be mediated by MEKK1-MKK1/MKK2-MPK4 pathway (Pitzschke et al., 2009).

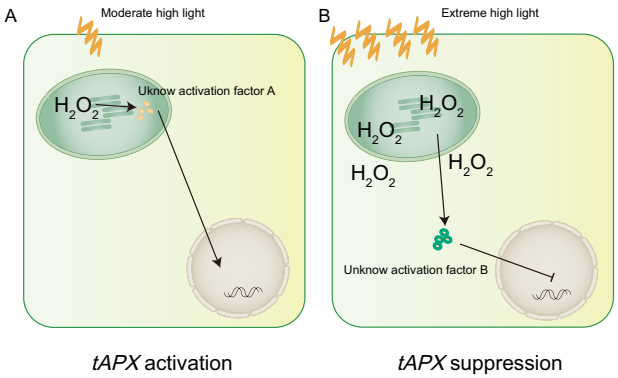

Figure 4.1 *tAPX* is moderated by H_2O_2 production and excessive H_2O_2 production. A: Moderate H2O2 productions stimulated by moderate light activate the *tAPX* transcription. B: extreme high light makes larger amount of H_2O_2 production. It also leaks to cytosol and stimulates *tAPX* suppression factors production.

4.3 Light intensity regulation of *tAPX* promoter

The production of chloroplastic H_2O_2 varies with the light intensity. When plants are subjected to high light illumination the electron transport is more active than lower light exposed plants. Consistent with that *tAPX* promoter is regulated with chloroplastic H_2O_2, light intensity dependent regulation of *tAPX* promoter was observed (Section 3.3.4). In the terms of *tAPX* promoter regulation, plants sense the light intensity via chloroplast H_2O_2 production. Accordingly, the activity of *tAPX* promoter increase when plants are subject the dark/light switch, a phenomenon intimating to night/dawn switch, the. Stimulation of *tAPX* promoter may come from the sudden increase of chloroplastic H_2O_2.

However, the *tAPX* promoter driven GUS measure also demonstrated that the regulation of *tAPX* promoter by light is more complex than a linear relationship. Figure 3.6 A and Figure 3.7 also showed that *tAPX* promoter stayed unchanged or suppressed when the plants were high light treated. The inert and suppression of the *tAPX* were observed when the plants were exposure to higher light intensity (400 or 1000 µmol photons m^{-2} s^{-1}). It seems that there is a threshold of the promoter regulation by light. Under moderate light illumination (moderate H_2O_2 production in chloroplasts) the *tAPX* promoter is activated while in excessive high light *tAPX* promoter activity is either irresponsive or suppressed. Under continues light condition, even when the light is moderate, *tAPX* promoter activity is suppressed (Figure 3.8). This suggested the responses of *tAPX* promoter to light/chloroplastic H_2O_2 need a dark interval period, which might be important for the renewal of the yet unknown signal molecules.

4.4 Light spectrum regulation of *tAPX*

Light spectrum response study showed that the promoter activity of *tAPX* is up-regulated by blue light and far-red light (Figure 3.9). Since the intensity of GUS activity did not recover to the level of plant grown under constant light condition (Figure 3.9), the recovery of *tAPX*

promoter activity by mono-spectrum light was a slow process. The recovery of photosynthestic activity, however, is as fast as few or tens of minutes (Gabrielsen et al., 1961; Baker and Butler, 1976). It is difficult to distinguish the regulation of photoreceptor- and photosynthesis-mediated regulation, because of the overlap of absorption spectra for photoreceptor and photosynthetic pigments (Lin, 2002). However, since the *tAPX* promoter could be activated by far red light (peak at 745 nm), the wavelength beyond the photosynthetically active radiation (400 – 700 nm) (Barnes et al., 1993), suggesting that the observed *tAPX* promoter regulation by far-red light is mediated by photoreceptors. It is unclear yet whether the blue light and red light response of *tAPX* promoter was due to the phytochrome, cryptochrome, or/and other phytoreceptors. Results in Figure 3.9 fit the scheme that blue light and far-red light have the same effect while red light and far-red light have the opposite effect on downstream gene expression (Figure 4.2).

Data taken from a group of microarrays experiments in the photoreceptors signaling mutants (*phyAphyB, cop1/det1,* and *pifs*, see following argument) support that the light spectra regulation of *tAPX* promoter is meditated photoreceptors (Schroeder et al., 2002; Devlin et al., 2003; Ma et al., 2003; Mazzella et al., 2005; Leivar et al., 2009; Oh et al., 2012). mRNA levels of *tAPX* were shown to be higher in *phyAphyB* mutant than wild type plants (Mazzella et al., 2005). Consistently, when the phyA and phyB genes were strongly up regulated, the *tAPX* expression rapidly went down (Devlin et al., 2003). COP1 and DET1 acts downstream of photoreceptors (Figure 4.2). *tAPX* expression is higher in both *cop1* and *det1* than wild type (Schroeder et al., 2002; Ma et al., 2003). HY5 is a transcription factor whose activity is suppressed by COP1/DET1. However, neither *tAPX* is down in *hy5* mutant, nor is there a HY5 binding site in *tAPX* promter (Lee et al., 2007a; Zhang et al., 2011). COP1/DET1 also regulates the protein stability of PIFs. PIFs are bHLH family transcription factors physically interacting with phytochromes (Castillon et al., 2007). Research identified *tAPX* as a gene that is PIF-repressed (Leivar et al., 2009; Oh et al., 2012). Chromatin immunoprecipitation sequencing also revealed the binding of PIF4 to *tAPX* promoter (Oh et al., 2012). Thus the transcriptional regulation observed in Figure 3.9 is possibly mediated by PIF4.

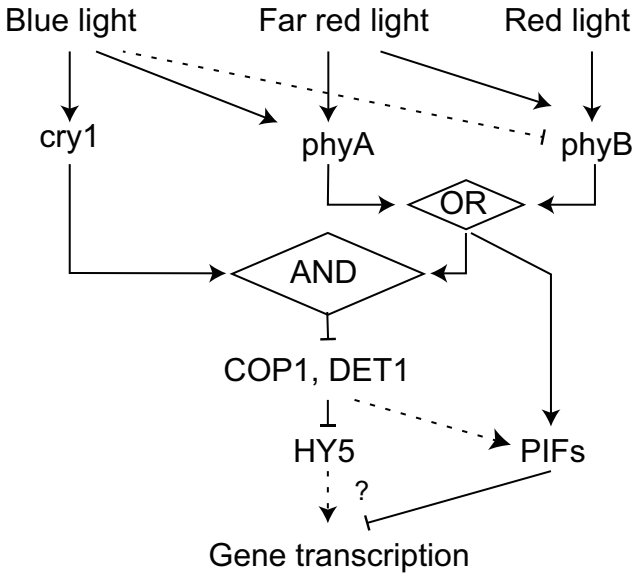

Figure 4.2 *tAPX* is regulated by photoreceptor. Cry1: cryptochrome; phyA: phytochorme A; phyB: phytochorme A; OR: antagonistic action; AND: syngeneic actions; COP1: constitutive photomorphogenic 1; DET1: de-etiolated 1; HY5: elongated hypocotyl 5; PIFs: photochromes interacting factors.Modified from Usami et al. (2004); Chen and Chory (2011).

A cyotosolic isoform, APX1, is also controlled by light receptor mediated regulation, via fast direct enzyme activation as well as transcriptional regulation (Kreslavski et al., 2013). In mustard (*Sinapis alba* L.), both non-plastidic (possibly cytosolic isoform) and plastidic APX are up regulated by red light irradiation, possibly mediated by phytochromes (Thomsen, 1992). The exposure can be harmful, but it is not necessarily the case. The perception of light renders plants prepared for the future stress. Thus, after treatment of red light, plants are less susceptible to photooxidative stress (Kreslavski et al., 2013). The transcriptional regulation of tAPX provides a good example on how plants combine the chloroplast-to-nucleus and the cytosol-to-nucleus signals.

4.5 Phytohormone regulation of *tAPX* promoter

tAPX promoter activity was not changed by SA application, while down regulated and up regulated by JA and ABA, respectively (Section 3.3.3). This indicated the activation or suppression is independent of cytosolic H_2O_2. Actually the exotic treatment by H_2O_2 will suppress the plants *tAPX* promoter. *tAPX* expression regulation by SA and ABA are specific rather than a secondary effect by H_2O_2 production.

4.6 Wounding regulation of *tAPX*

During the process of studying the tissue specificity of promoter activity of *sAPX* and *tAPX* gene, wounding activation of *tAPX* promoter was observed. Further studies showed a strong wounding activation of *tAPX* promoter in young leaves, but not in the older leaves (Figure 3.5). Wounding stimulates a verity of signaling molecules including H_2O_2, phytohormones, oligosaccharides, and protons *etc.* (Leon, 2001). Wounding triggering H_2O_2 production of

ROS is confined to cytosol and extracellular matrix (Sagi et al., 2004). As the activation of *tAPX* promoter is independent of H_2O_2 production outside chloroplasts, the *tAPX* promoter activation observed here is mediated by other signals. Accordingly, disassociation of wounding triggered H_2O_2 production and *tAPX* promoter activity was observed in older leaves, where the wounding triggered H_2O_2 production was seen while *tAPX* promoter was not activated (Figure 3.5). Other possible factors are the phythormones. The JA and SA were shown acting downstream of wounding effect (Leon, 2001). However, directly treatment of neither JA nor SA induced *tAPX* promoter, indicating the wounding stimulation of *tAPX* is not mediated by JA or SA. The induction of the *tAPX* promoter by wounding was a fast process (1 hour), while the hormones biosynthesis and signaling triggered by wounding is much slower (around 2 hours) (Creelman et al., 1992; Niki et al., 1998). As the *tAPX* is regulated by a combination of chloroplast and cytosolic signal cascade, it is tempting to speculate that the regulation can be modified by other factors, such as wounding triggered cellular signals. How the wounding-effect connecting with the retrograde and anterograde signaling pathway is yet to be studied.

4.7 Transcription factor of SPL in regulating *tAPX*

In Arabidopsis, SPL family consists of 11 transcription factors, regulating a variety of processes, such as plant growth and development (Preston and Hileman, 2013). For the plant vegetative and productive growth transition, SPL is targeted and negatively regulated by microRNA (miR156), which is negatively regulated by age. And SPL in turn activates flowering promoting transcription factors AP1. The regulation of *tAPX* appears to fit in the regulation sheme. Firstly, *tAPX* expression is repressed with plant age. Second, the available of transcriptomic profiles of miR156 suggested that *tAPX* is positively regulated by this micro RNA. And more downstream, *tAPX* was shown in this research up-regulated in the tested *spl3* and *spl8* mutants. However, the two SPLs belong to the clade III SPL (Salinas et al., 2012), whose is not directly regulated by miR156 or miR157. Other micro RNA regulating SPLs may be involved in the regulation of *tAPX*. All SPLs bind to a conserve palindromic GTAC core motif, whose flanking sequence is essential for SPL binding preference (Birkenbihl et al., 2005). Thus further test is needed to maintain that SPL transcription factor incorporate the *tAPX* regulation into developmental control.

4.8 Distinct regulation pattern of *sAPX* promoter in mesophyll cells and leaf vasculature

The *sAPX* promoter driven reporter expression in leaf non-vascular tissue was not the detected by GUS staining (Figure 3.3). However, a more sensitive method measuring the GFP expression driven by the same promoter suggest that the *sAPX* promoter is active in the non-vascular tissue (Figure 3.3), but with lower activity than that in vasculature. Later experiments also suggested that *sAPX* promoter activity in vascular and non-vascular tissue is under different regulation (for example, Figure 3.18). For this reason, it is not possible to study the reporter activity measuring GUS enzyme activity using plant leaf extracts. Besides, the transcript level data of *sAPX* taken from microarray measuring mRNA of whole leaf extract should be used with caution.

By nature, the transcription of *sAPX* in plant photosynthetic tissue is very low, suggesting that sAPX is not involved in protecting the photosynthestic memembrane. If the protein level of sAPX is consistent with the transcript, there should be only subtle level of sAPX in the leaf blade. Western blot using polyclonal antibodies binding sAPX, tAPX, and APX1 showed that the band for sAPX is weaker than the bands for tAPX and APX1 from the same sample (Davletova et al., 2005). This is likely due to the low transcriptional activity of sAPX in the leaf blade. With such amount, the influence of sAPX to the photosynthesis is limited. *sAPX* knocked out plants did not show any adverse effect on photosynthesis neither accumulate H_2O_2 under controlled growth condition (Davletova et al., 2005). The authors assert that the function of sAPX in scavenging H_2O_2 in chloroplast stroma and mitochondria matrix is substituted by APX1 as H_2O_2 can diffuse out from the organelles (Davletova et al., 2005).

Promoter activity of *sAPX* in these photosynthetic tissues, however, was shown to be controlled by the choloroplast physiological state and photosynthesis, as the evidence is that chemical treatment causing chloroplast malfunction and blocked photosynthesis rendered lower *sAPX* promoter activity in plant (Figure 3.17 and Figure 3.20). The link between photosynthestic activity and *sAPX* promoter regulation is bridged by ascorbate biosynthesis, which relays on the carbohydrates synthesized in chloroplasts (Heiber et al., 2014). It was long observed that *sAPX* is regulated by the ascorbate pool size (Kiddle et al., 2003a). Feeding ascorbate does not change the redox state of ascorbate pool, namely the ratio between reduced and oxidized ascorbate. mRNA level of *sAPX* in leaves was shown up regulated by ascorbate pool size (Kiddle et al., 2003a). Feeding plant with sugar promoted ascorbate synthesis, and increased the *sAPX* mRNA level (Heiber et al., 2007; Heiber et al., 2014). The

sugar induced *sAPX* mRNA level was attributed to ascorbate biosynthesis, as the less induction effect by sugar was seen in *vtc1* mutant deficient in ascorbate biosynthesis (Heiber et al., 2014). To sum up, the transcription of *sAPX* in plant leaf blade is mediated by the acerbate level.

Besides this type of regulation, some peculiarities were observed for *sAPX* promoter, *i.e.* induction of *sAPX* expression in trichome by cold and blue light.

4.9 Cold regulation of *sAPX* promoter

Here, the results for *sAPX* promoter activity showed strong cold induction of this promoter (Cross reference). This is consistent with previous study of the mRNA level of plants under low temperature (Kangasjärvi et al., 2008). The authors also showed that the loss of function of *sAPX* did not cause a phenotypic difference for plants under cold. The promoter driven reporter assay (Cross reference) also showed a high promoter activity in trichome under cold stress. The trichome specificity may be a common pattern for a group of other cold inducing genes. *COR* (cold regulated), *KIN* (cold induced), *LTI* (low temperature induced) or *RD* (responsive to desiccation) genes are known low temperatures genes (Medina et al., 1999). Promoter fused GUS assay also showed the trichome localization of Kin1 and Cor6.6 promoter (Wang and Cutler, 1995). Using microarrays, (Jakoby et al., 2008) studied the Arabidopsis trichome specific expression genes. In his list of highly expressed genes in mature Arabidopsis trichomes, LTI30, RD19A, RD29A, and COR15B is found. These studies suggest that trichome expressed genes may have an important role in plant cold response. It is hard, however, to relate the high transcription of *sAPX* in trichomes to its function in chloroplasts, as the trichome cells are thought not to contain chloroplasts (Jakoby et al., 2008). COR15B is also a chloroplastic protein (Lin and Thomashow, 1992), whose function is to stabilized the chloroplast membrane while the cold stress (Thalhammer et al., 2014). The expression of COR15B was found in trichome (Jakoby et al., 2008). Although intriguing, the similarity of cold response of *sAPX* and other cold regulating genes shed little light on the possible role of *sAPX* in trichome under cold stress, since the function of these COR/KIN/LTI/RD is largely unknown. Compared with the cold suppression effect on tAPX promoter, the activation behavior of *sAPX* provides another line of evidence that *sAPX* and tAPX have distinct function in the plant cell.

4.10 Light regulation of *sAPX* promoter

The analysis of *sAPX* promoter reporter gene lines demonstrated a distinct response of *sAPX* promoter to high light in leaf vasculature and mesophyll cells (Figure 3.3). Up-regulation of *sAPX* promoter activity was seen in leaf mesophyll cells by high light (Figure 3.3). Other researchers measuring mRNA also suggested that *sAPX* is a high light response gene. Kimura et al. (2003) suggested that *sAPX* is high light response gene based on a cDNA microarray study. *sAPX* was found to be the only *APX* responding to high light in the experiment of Hebbelmann et al. (2012). Clearly, the high light response is related to the chloroplast physiological function because the *sAPX* promoter activity can be modified by using the photosynthetic electron transport chain blocker or other chemicals causing chloroplasts malfunction (Figure 3.17 and Figure 3.20). H_2O_2 might be the signal mediator to reflect the chloroplast physiological function, as direct erogenous addiction of H_2O_2 increased the *sAPX* transcripts supports the direct involvement of H_2O_2 production in regulating *sAPX* promoter (Moffat, 2007; Sukrong et al., 2012). Nevertheless, the transcripts analysis of *APX* family genes in the *apx1* knockout mutant stands against this view. Enhanced production of H_2O_2 in *apx1* did not cause a change of the *sAPX* mRNA level (Davletova et al., 2005).

However, light suppression of *sAPX* was observed in the plant vasculature (Figure 3.3). The light repression (or dark activation) of plant gene promoter is quite common in plant. An example is that the presence of GT-elements (GCGGTAATT) rice PHYA gene mediates light-repression of its expression (Dehesh et al., 1990). Actually, there are also 13 GT-elements in the -95 to -630 bp promoter region of *sAPX* promoter (Supplenmenatry 4-A). The light repression of *sAPX* in vasculature may be likely mediated by GT transcription factors.

4.11 Light receptor regulation of *sAPX* promoter

The dotted pattern of *sAPX* promoter indcution, higher expression of *sAPX* in trichomes, suggests that blue light regulation is distinct from the regulation by photosynthesis/ascorbate signal (Figure 3.19), since the photosyntsis/ascorbate regulation of *sAPX* was seen in leaf blade. The activation is blue light specific (red light and far red light are not able to cause such induction, see Figure 3.19). So far, the significance of the blue light induction of *sAPX* in trichomes are not clear. Like the blue light regulation of *tAPX*, the regulation of *sAPX* can also be confirmed by data from microarray study. Under white light condition, *sAPX* expression was not changed in the *phyAphyB* mutant. However, expression was elevated in the *cry1cry2* mutant (Mazzella et al., 2005). The elevation was suppressed by *phyAphyB*

mutantion in *cry1cry2* background (Mazzella et al., 2005). The *sAPX* is regulated through a cross-talk signal from phytochrome and cryptochrome mediated signal.

Thomsen et al. (1992) for the first time reported that the light receptor mediated regulation of chloroplastic APX. The authors deduced that the observed elevation of enzyme activity was attributed to the *de novo* synthesis of the protein. However, it remains obscure which chloroplastic isoform (sAPX or tAPX) is regulated and which expressional step(s) is regulated by photoreceptor mediated signal. This study suggests that both *sAPX* and *tAPX* are transcriptionally regulated by blue light. Blue light mediates a series of important process important for chloroplasts, such a chloroplast development, chloroplast high-light avoidance movement (Kagawa et al., 2001). Actually, many chloroplast localized proteins are both regulated by photosynthesis and photoreceptor mediated signal (Pfalz et al., 2012; Mellenthin et al., 2014). It is now accepted that photoreceptor mediated signal and chloroplastic signal can work together on the same promoter, while photoreceptor mediated signal are especially important for the "build-up" of the chloroplasts (chloroplast development) while chloroplasts originated signal is critical for "functional control" (Pogson et al., 2008; Pfalz et al., 2012).

Furthermore, as chloroplast APX are capable to modify the amount of chloropalstic H_2O_2, which can work as gene regulation signal, they control genes expression. mRNA levels of *CRY2* is modified in *sapx* and *tapx* mutants (Kangasjärvi et al., 2008). Thus the regulation relation of chloroplast APX and blue light receptor is a closed circuit. *i.e.* chloroplast APXs modifies the abundance of blue light receptor, while the blue light receptors mediate the regulation of chloroplastic *APX*.

5 Supplementary

Supplementary Data 1 Promoter sequences in obtained *pro*sAPX:EGFP-GUS and *pro*tAPX:EGFP-GUS constructs

sAPX promoter sequences (1947 bp) in *pro*sAPX:EGFP-GUS construct

TTTCAGCCATCTGGTCGTCGTCCGTTATATGGATGCTATCGTTTTAACATTGTAATTACCGATTATCTTTTTTGTTGAATAAATTTTAAAT
promoter starts
GCTTAGGACAAAACTAATATTACATGTTGTGGTTTCGGACCTGGAGAGATTACAGGCTTCGGCCCAAAACTTCCTTGTATTAAAAAAA
AAAAAAGAATAAATTTTAAATTCTAAATAAAAAAGATATTTACTAAGGATTTTTGTGAAAGCAATACTAATTGCATTAACATTCATACATGAT
TTGTATAATTGTTTGGAGTTGGGTAAGATGCTCGACTTCCACATTAGGGAACAAACAAAGCTAGATTAATGCACGTCTAGTGAAAGAT
CCATCCCTTTGATTACTTAACTTCACATTAGGGAAAAAACAAAGCTAGGTTCATGCACTTCTAGTGAATGATCCATCCCTTTGATTACTT
AACTTCACATTTAGGGAACAAACAAAGCTAGATTCATGCACTTCTAGGTGAAGGATATCAGGATATGCCACATTTCTATCAAAGAATAC
GGTTAAGAAAGTCTATAGGAGGACAAAAATAGATATGTTTTCCCAAAAAAGATTCAAAGCTCAAGTTAAATGGAACAATGAGTTAGCAA
AAAATGGTTTAACGGGAGGAGAAATAGTGGTAGTGTCAACCAAGTCGCCTTGGTCCAGTAGTAAAGAGAGGTAATTCCTGTCACATG
GGTTCGAGTAGCTTTGGCCACTGGGATTCGAGTAATTTAACATGGGTTTTTTCGGGTTCCAGAATTAGTCTTTGGACCTAGAAACCCT
TTAAGATCACCTGAATTATCAAAAAATATATATATAGTAGTAGTGTTGTGTCCTATGTTGAAAAGTAATATGGATCAGCTTTGGAATTCTC
CAGGAAGCAATGGGATCATGTGGGATCAAAAAGAATAATAAAGAGACCACGATTGGGCAATTACTAGGCACTTATGTCATTGAGGTGA
TTAGTGGATCATAAGATTATGCCTACTGTTATCCTCAATTTGACCGTATACTATTTTCTTCTCTTTTAGCCATCCTTGAATACCTAACCGA
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TTTTTTTTTTCTCTTTAATTAAAATAGGATTGTCTCTAAAATAAAATTTTACCTAATCCCCGATTTTGTAATTTTACTTTTCATCTCAAAGAA
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AATCAAAACTTTCGTGATGCAGAATTCAATCTTTAGAATTTTAAACATCTAATACATATGATCGTAATAAATTTTAGAATATACATATAAAAA
TGCATATTATTTTTTACATAATAATATCGATTTCCGTTGTTTACTATACACGTAGCAAGACCTAAACAGTTTTAATCTGATATAGCGGAAAC
TAATTTTGGCACCTTAGACAATTGATTGGTCAATTCAACCCTCAATAAGCCCAAGTGGAAAGACAAAGAAATTTAGGGTATTTTGTCGA
ATGAGACATAATCAACTTGACTGCTCCAATAAACTGTTTTCACTAGATGATGGATAGTAGTTCTGTTCTAAACATGGTTAATAGAGATCC
AAAACCCAATTTCTCTATGGACTTTATTGGTGCAACTCTTTAAGTGAAATTGAAACCTTACTTCAACACATTTCAAATGTATCTACCTAAT
TAACTCTTTATATAAAATTAAAAAAAACCTTAGTGCATAAGTGGGTAGGTGAACTTGAGTCTCTTGGCAATATGTAAAGAGGCGAAATC
AAAGTTGATCAACAATTAAACACAAAAACCCTTAAGTTGACGTGGCTGTATCTCTtccaactcttaaaaatcaattactattatacccctcagaaaaATG
Sequences highlighted in yellow: 5'-UTR
promoter ends
Uncloned promoter sequences
Translation start codon

tAPX promoter sequences (1519 bp) in *pro*tAPX:EGFP-GUS construct

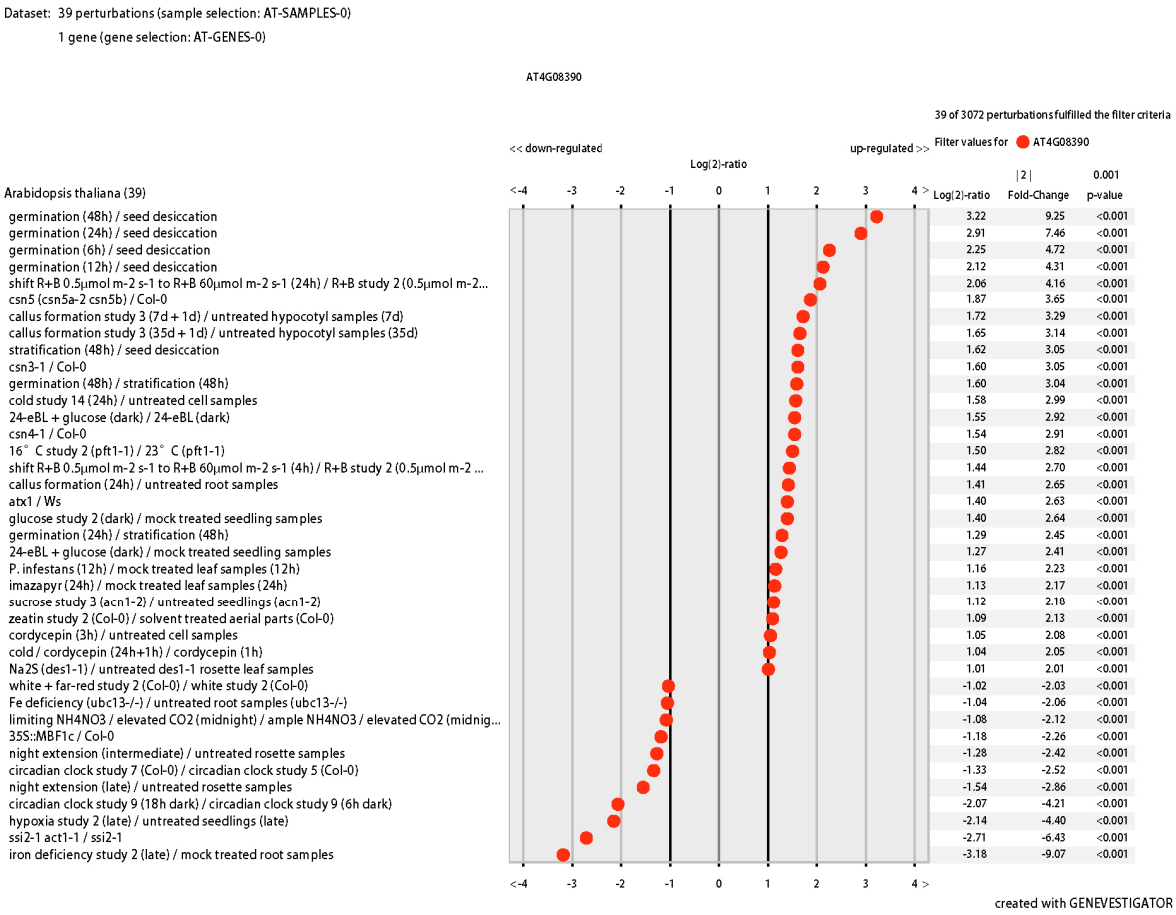
ATCAGTTACAAGTGCACCAATAGATGATGCATGATCGGAGTATCCGATTTCATAGTCACAATCAGTTACAAGTGCACCAATAGATGATG
promoter starts
CATGATCGGAGTATCCGATTTCATAGTTGATCCTCAGGGTCAGCACAAGGTCTGTCCTGGGGAAGGTCAAGACCAGAACACAAGAT
GTGTGAACAAGGCAAAGTGTTGTGATTGGGCTTGTACTGCTTAGCACGAGGCTGCAAAGAAAGAGAAAGAAATTCTAAGATAGAACC
TTTGCTTATTATGTCACACACTAAAAAGCATATCAACGCTTACCTTTGTGCAACCGTTGCAAGGTGCATCACACTGAACCCAAGTGAG
GTCACTGCCAGTGTCAATGTCCAAGTCAAAGAGCTTAGGTGGGTTGCCAATGTTAAGCAACACATAATAGTACCTGCAAAGATGAATT
CGTTAATTACTTCAACAATCAAATTGGGCGTTTTCAATACAAGGTGAAAATCAGAATCAACATTGCCCAAAATCTTCAATTCGTGAAAG
TAGCAAGCTTTGAAATCATCTCAAAGATCAATAGAATGAAGATTTCACATCTTGTCAAGCAATGAAATAGATTACAAGTGAAGAATCCA
CACAGATGCAAGAGAAGCCAACAAAGTATCCGCACCAATTCAAAATCCAGACATAAGTCAACACAATCCCGTTAAGGAAAATCAAAG
AAACAAGATCAAACCCACGAATGGGTCACTGAAAAGTAGCATAAAACAGAGAAATTGAAACAAAAAGATGCGAAATTTGGCACTTAC
CCAAGAGGATACACATTCCCGGAGACAGGAAACACGACGGTGGAGCTAAGTCGACGATTCTGTAACTTCACTTGAGCCGACGAGTC
TTTAGTAGCTTCAGAGGTTTGAAAGCGAGCACAGAGGATGAAGACATAGAAGACGAAGACGAAGAAGAAGTAAAGGTTTCTTCTTTG
CTGCTTATTCGGATTCATCATCTCCTCTTTTTGGCCCCCAAGAAAGCGAAACCAAATCCCACCAAAAAGGTTTCTTTTTTGACACAGG
GCAACAAAAAGGAGGCTTTTTCTTTGTAGAATTGCTGCTCCCTTATAGGAGTGAGTATTTGTTTATATATCTGATCTCTCTCACACTCAA
TTTCGTGCAACTTGCAGCAGAGGTTTCTTTGAGTTCCCGTAAGCTAAATTGAGAAACTTGGCATTTGGCTAAAAGAAACTCAACGCC
CAACGCTCACTACACTTAGCGTGTAAAGCACGCGCCAACCAGGATTGACTCAAAGGCCTAACGATCTTCAAATGTACAGGGCCCAAT
ATACTCGGCCCATAAACTCTAAAACTATTCAAATGTTAGGCGCGTGACGTTCACCTGTGAATCAGCTGATAGAAATCATTATCCAATAT
CCAGTTATGAGTGGGTGACTCCAAATAGATCCAACTGGCAACGCATTATTGACACGTGTCTTTTCATTTCTCTTCTTCCGGCGTTTTC
TCTCCGCCGTTTCGCCACCGTACGTGAcaATG
promoter ends
Uncloned promoter sequences
Translation start codon
Sequences highlighted in yellow: 5'-UTR

Supplementary Data 2 T-DNA lines used in this study and primers used for verification

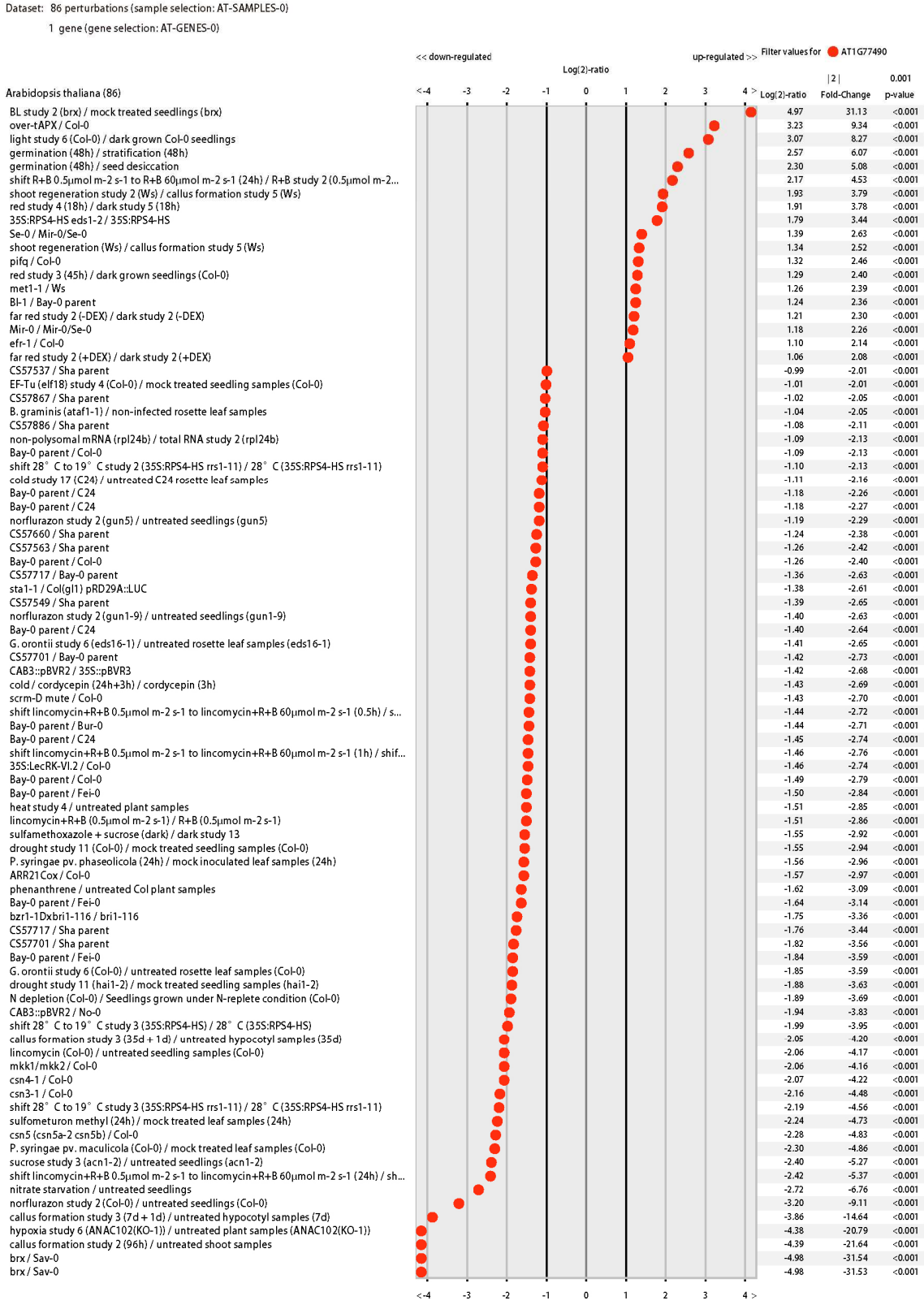
Name	Gene	T-DNA lines	Primers design for verification	Primers design for RT-PCT
arr2-3	AT4G16110	SALK_043107	CAATAGACCAAAACCATAAAAGGATACGA	ATGGTAAATCCGGGTCACG
			AATAACAGAATCAGAGTCAAAAAGCATCAA	TCAGACCTGGATATTATCGATG
arr10	AT4G31920	SALK_025664	CTTACATGGAAGCACTCGGAG	ATGACTATGGAGCAAGAAATTGAA
			TCAGAGTGGTTTGATGGCTTC	TCAAGCTGACAAAGAAAAGG
spl3-1	At2G33810	SALK_035860	CAAACCTCCTTGCACGTTAAG	ATGAGTATGAGAAGAAGCAAAGC
			TAAAGGAGCCCCAAACAAAAG	TTAGTCAGTTGTGCTTTTCCG
spl3-2	AT2G33810	SALK_035917	CAAACCTCCTTGCACGTTAAG	ATGAGTATGAGAAGAAGCAAAGC
			TAAAGGAGCCCCAAACAAAAG	TTAGTCAGTTGTGCTTTTCCG
spl8	At1G02065	SAIL_816_E01	GAGAGGGTTCGTACTGTGTGG	ATGTTGGACTACGAATGGGAT
			GGACGTACATTGATCGGTCAC	CTATCCGCTGGAGAAAAACAT

Note: Primers for homozygosity verification were designed according to (Salk Insititue Genomic Analysis Laboratory, 2003); primers designed for RT-PCR detection was according to

Supplementary Data 3A Perturbtions change *sAPX* expression by >2 folds, $p \leq 0.001$



Supplementary Data 3B Perturbtions change *tAPX* expression by >2 folds, $p \leq 0.001$



Supplementary Data 4A List of potential *cis*-regulatory elements in *tAPX* promoter

cis-elements, signal sequence, description and reference are listed.

cis-element	Sequence	Description	Reference
AGATCONSENSUS	TTWCC(W)^{4}NNGGWW	Binding consensus sequence for the product of the Arabidopsis floral homeotic gene AGAMOUS; W=A/T; N=any base ((not a gap));	(Mizukami et al., 1996)
ARF1	TGTCTC	Binding site found in the promoters of primary/early auxin response genes of Arabidopsis thaliana, response to IAA and BL;	(Goda et al., 2004)
CARGCW8GAT	C(W)^{8}G	Binding site for AGL15; (W)^8=WWWWWWWW;W=A/T;	(Tang and Perry, 2003)
DRE/CRT	RCCGAC	Response to drought; high-light; cold;	(Suzuki et al., 2005)
HEXAMERATH4	CCGTCG	Hexamer motif of Arabidopsis histone H4 promoter;	(Chaubet et al., 1996)
LEAFYATAG	CCAATGT	Target sequence of LEAFY in the intron of AGAMOUS gene in Arabidopsis;	(Lohmann et al.)
MYB1AT	WAACCA	MYB recognition site found in the promoters of the dehydration-responsive gene rd22 and many other genes in Arabidopsis; W=A/T;	(Abe et al., 2003)
MYB1LEPR	GTTAGTT	Tomato Pti4(ERF) regulates defence-related gene expression via GCC box and non-GCC box cis elements (Myb1(GTTAGTT), G box (CACGTG));	(Chakravarthy et al., 2003)
MYB2AT	TAACTG	Binding site for ATMYB2; Response to water stress in Arabidopsis;	(Urao et al., 1993)
RAV1-B	CACCTG	Binding consensus sequence of an Arabidopsis transcription factor, RAV1; RAV1protein contain AP2-like and B3-like domains; The expression level of RAV1 were relatively high in rosette leaves and roots;	(Kagaya et al., 1999)
TBOXATGAPB	ACTTTG	"Tbox" found in the Arabidopsis GAPB gene promoter; Mutations in the "Tbox" resulted in reductions of light-activated gene transcription; GAPB encodes the B subunit of chloroplast glyceraldehyde-3-phosphate dehydrogenase(GADPH) of Arabidopsis;	(Chan et al., 2001)

Supplementary Data 4B List of potential cis-regulatory elements in *sAPX* promoter

Cis-elements, signal sequence, description and reference are listed.

***cis*-element**	**Sequence**	**Description**	**Reference**
AACACOREOSGLUB1	AACAAAC	Core of AACA motifs found in rice glutelin genes, involved in controlling the endosperm-specific expression; AACA is also closely associated with the GCN4 motif in all rice glutelin genes and together have been shown to confer endosperm-specific enhancement to the truncated -90 CaMV 35S promoter;	(Wu et al., 2000)
ARF1	TGTCTC	Binding site found in the promoters of primary/early auxin response genes of Arabidopsis thaliana, response to IAA and BL;	(Goda et al., 2004)
GT1CORE	GGTTAA	Critical for GT-1 binding to box II of rbcS; GT1MOTIF1; For a compilation of related GT elements and factors,	(Villain et al., 1996)
MYBATRD22	CTAACCA	Binding site for MYB (ATMYB2) in dehydration-responsive gene,rd22; MYB binding site in rd22 gene of Arabidopsis; ABA-induction; Located at ca. -141 of rd22 gene; Also MYC at ca. -200 of rd22 gene;	(Abe et al., 1997)
MYCATRD22	CACATG	Binding site for MYC (rd22BP1) in Arabidopsis dehydration-resposive gene, rd22; MYC binding site in rd22 gene of Arabidopsis; ABA-induction; Located at ca. -200 of rd22 gene;	(Busk and Pages, 1998)
PREATPRODH	ACTCAT	"PRE (Pro- or hypoosmolarity-responsive element) found in the promoter region of proline dehydrogenase (ProDH) gene in Arabidopsis; Core of 9-bp sequence ACTCATCCT which is necessary for the efficient expression of ProDH in response to L-Pro and hypoosmolarity; ATB2-binding site; Similar to GCN4 motif (ATGA(C/G)TCAT); ATB2 subgroup of bZIP transcription factors function as transcriptional activator for hypoosmolarity-inducible ProDH;	(Satoh et al., 2002) (Satoh et al., 2004)
RAV1AAT	CAACA	Binding consensus sequence of Arabidopsis transcription factor, RAV1; RAV1 specifically	(Kagaya et al., 1999)

		binds to DNA with bipartite sequence motifs of RAV1-A (CAACA) and RAV1-B (CACCTG); RAV1 protein contain AP2-like and B3-like domains; The AP2-like and B3-like domains recognize the CAACA and CACCTG motifs, respectively; The expression level of RAV1 were relatively high in rosette leaves and roots;	
RAV1-B	CACCTG	Binding consensus sequence of Arabidopsis transcription factor, RAV1; RAV1 specifically binds to DNA with bipartite sequence motifs of RAV1-A (CAACA) and RAV1-B (CACCTG); RAV1 protein contain AP2-like and B3-like domains; The AP2-like and B3-like domains recognize the CAACA and CACCTG motifs, respectively; The expression level of RAV1 were relatively high in rosette leaves and roots;	(Kagaya et al., 1999)
SORLIP2AT	GGGCC	One of "Sequences Over-Represented in Light-Induced Promoters in Arabidopsis; Computationally identified phyA-induced motifs;	(Hudson and Quail, 2003)
TBOXATGAPB	ACTTTG	"Tbox" found in the Arabidopsis GAPB gene promoter; Mutations in the "Tbox" resulted in reductions of light-activated gene transcription; GAPB encodes the B subunit of chloroplast glyceraldehyde-3-phosphate dehydrogenase(GADPH) of Arabidopsis;	(Chan et al., 2001)
ELRECOREPCRP1	TTGACC	Elicitor Responsive Element core of parsley PR1 genes; consensus sequence of elements W1 and W2 of parsley PR1-1 and PR1-2 promoters; Box W1 and W2 are the binding site of WRKY1 and WRKY2, respectively; ERE; "WA box"; One of the W boxes found in the Parsley WRKY1 gene promoter; Required for elicitor responsiveness; "WC box" WB box and WC box constitute a palindrome; WRKY1 protein binding site; W-box found DE in thioredoxin h5 gene in Arabidopsis (Laloi et al.);	(Chen and Chen, 2000; Rushton et al., 2002; Laloi et al., 2004)
MYB1AT	WAACCA	MYB recognition site found in the promoters of the dehydration-responsive gene rd22 and many other genes in Arabidopsis; W=A/T;	(Abe et al., 2003)
MYBPLANT	MACCWAMC	Plant MYB binding site; Consensus sequence related to box P in promoters of	(Sablowski et al., 1994)

		phenylpropanoid biosynthetic genes such as PAL, CHS, CHI, DFR, CL, Bz1; Myb305; M=A/C; W=A/T;	
MYCATERD1	CATGTG	MYC recognition sequence (from -466 to -461) necessary for expression of erd1 (early responsive to dehydration) in dehydrated Arabidopsis; NAC protein bound specifically to the CATGTG motif ; NAC protein bound specifically to the CATGTG motif;	(Simpson et al., 2003; Tran et al., 2004)
SV40COREENHAN	GTGGWWHG	"SV40 core enhancer"; Similar sequences found in rbcS genes; W=A/T;	(Weiher et al., 1983; Green et al., 1987; Donald and Cashmore, 1990)
MYB4 binding site motif	AACTACC	Expression profile matrix of Arabidopsis transcription factor genes suggests their putative functions in response to environmental stresses.	(Chen et al., 2002)
MYB4 binding site motif	ACCTAAC	Expression profile matrix of Arabidopsis transcription factor genes suggests their putative functions in response to environmental stresses.	(Chen et al., 2002)

Supplementary Data 5A The putative transcription factor binding sites in *tAPX* promoter -1521 bp to -21 bp promoter region.

Factor	Family	Number of TFBS	Upstream orientation	
			+	-
AG	MADS	1	0	1
AGL1	MADS	1	0	1
AGL15	MADS	1	1	0
ALFIN1	HD-PHD	5	2	3
ARR10	**GARP**	**1**	**0**	**1**
ARR2	**GARP/ARR-B**	**1**	**0**	**1**
AtLEC2	**ABI3/VP1**	**3**	**1**	**2**
AtMYB61	MYB	1	0	1
AtSPL3	**SBP**	**2**	**1**	**1**
AtSPL8	**SBP**	**2**	**1**	**1**
bZIP_DOF	- other	2	2 (both strands)	
DOF2	C2C2(Zn) Dof	12	10	2
GAMYB	MYB	1	0	1
GT1	Trihelix	3	2	1
HOX2a_HOX2a	- other	1	1 (both strands)	
HVH21	HD-KNOTTED	4	0	4
MYB46	MYB	2	0	2
MYB83	MYB	2	0	2
NtERF2	AP2/EREBP	1	0	1
O2	bZIP	2	1	1
P	MYB	1	0	1
RAV1(1)	AP2/EREBP	3	3	0
TaMYB80	MYB	1	1	0
TaNAC69(2)	NAC	1	0	1
TEIL	AP2/EREBP	3	1	2
TGA1a	bZIP	2	1	1
ZmHOX2a(1)	HD-HOX	2	2	0
ZmHOX2a(2)	HD-HOX	3	2	1

Note: The Transcription factors which is not presented in table 1b is mark in bold characters.

Supplementary Data 5B The putative transcription factor binding sites in *tAPX* promoter -1521 bp to -21 bp promoter region.

Factor	Family	Number of TFBS	Upstream orientation	
			+	-
AGL1	MADS	1	0	1
AGL15	MADS	2	1	1
AGL3	MADS	1	0	1
ALFIN1	HD-PHD	2	1	1
ANAC81	NAC	1	1	0
AtMYB61	MYB	1	1	0
bZIP_DOF	- other	2	2 (both strands)	
CBF	- other	3	1	2
CBP60g	CAMTA-like	1	1	0
DOF2	C2C2(Zn) Dof	19	11	8
GAMYB	MYB	3	3	0
GT1	Trihelix	1	1	0
HOX2a_HOX2a	- other	4	4 (both strands)	
HVH21	HD-KNOTTED	5	4	1
MYB46	MYB	2	2	0
MYB83	MYB	2	2	0
NtERF2	AP2/EREBP	10	5	5
O2	bZIP	2	1	1
P	MYB	2	2	0
PCF2	TCP	1	1	0
RAV1(1)	AP2/EREBP	2	2	0
RAV1(2)	AP2/EREBP	2	1	1
SARD1	CAMTA-like	1	1	0
TBF1	HSF	1	1	0
TEIL	AP2/EREBP	3	0	3
TGA1a	bZIP	4	2	2
TSS	- other	2	1 (both strands)	
ZmHOX2a(1)	HD-HOX	7	1	6
ZmHOX2a(2)	HD-HOX	4	2	2

Note: The Transcription factors which is not presented in table 1b is mark in bold characters.

Supplementary Data 6A The putative transcription factor and small RNA binding sites in *sAPX* promoter -654 to -21 bp promoter region.

Factor	Family	Number of TFBS	Upstream orientation	
			+	-
AGL15	MADS	4	2	2
AGL2	MADS	1	0	1
AGL3	MADS	2	1	1
AGP1	GATA	2	1	1
ALFIN1	HD-PHD	1	0	1
AtMYB61	MYB	1	0	1
bZIP_DOF	- other	1	1 (both strands)	
CBF	- other	1	1	0
DOF2	C2C2(Zn) Dof	9	5	4
GAMYB	MYB	2	1	1
GT1	Trihelix	13	3	10
GT-1	Trihelix	1	1	0
HVH21	**HD-KNOTTED**	**1**	**0**	**1**
ID1	C2H2(Zn)	1	0	1
MYB46	MYB	4	1	3
MYB83	MYB	4	1	3
NtERF2	AP2/EREBP	1	1	0
O2	**bZIP**	**1**	**0**	**1**
P	MYB	2	1	1
smallRNA(fl3)	small RNAs	8	5	3
smallRNA(i)	small RNAs	3	0	3
smallRNA(i2)	small RNAs	5	1	4
smallRNA(le3)	small RNAs	10	1	9
smallRNA(s2)	small RNAs	3	2	1
smallRNA(se3)	small RNAs	6	4	2
smallRNA(si3)	small RNAs	14	5	9
TBP	- other	1	0	1
TEIL	AP2/EREBP	5	3	2
TGA1	**bZIP**	**1**	**0**	**1**
TSS	- other	1	0 (both strands)	
WRKY26	WRKY(Zn)	2	1	1
WRKY38	WRKY(Zn)	1	0	1
WRKY43	WRKY(Zn)	1	0	1
ZmHOX2a(2)	HD-HOX	2	1	1

Note: The Transcription factors which is not presented in table 1b is mark in bold characters.

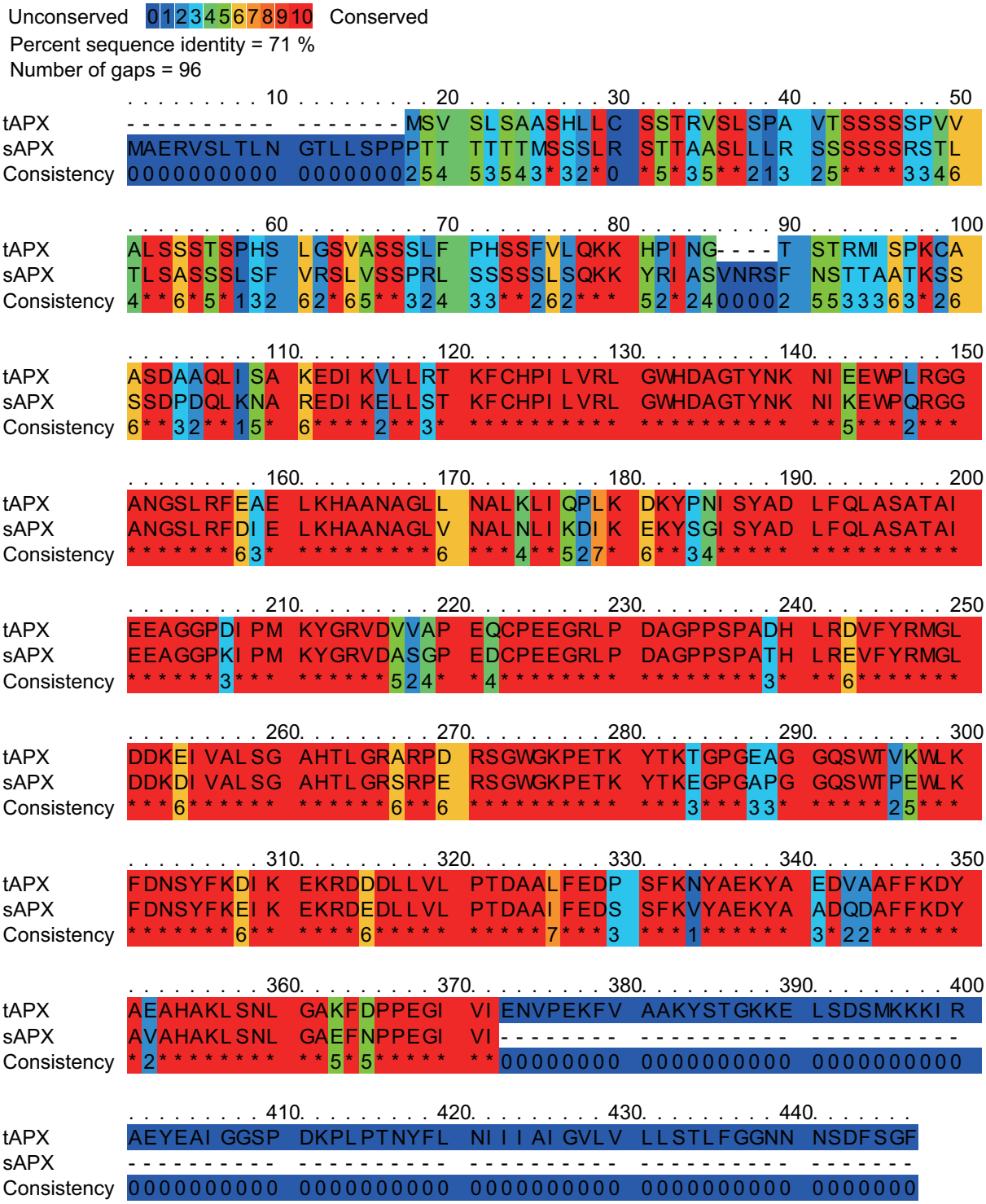
Supplementary Data 4B The putative transcription factor small RNA binding sites in *sAPX* promoter -378 to -21 bp promoter region.

Factor	Family	Number of TFBS	Upstream orientation	
			+	-
AGL15	MADS	4	2	2
AGL2	MADS	1	0	1
AGL3	MADS	2	1	1
AGP1	GATA	2	1	1
ALFIN1	HD-PHD	1	0	1
AtMYB61	MYB	1	0	1
bZIP_DOF	- other	1	1 (both strands)	
CBF	- other	1	1	0
DOF2	C2C2(Zn) Dof	7	4	3
GAMYB	MYB	1	1	0
GT1	Trihelix	6	2	4
GT-1	Trihelix	1	1	0
ID1	C2H2(Zn)	1	0	1
MYB46	MYB	4	1	3
MYB83	MYB	4	1	3
NtERF2	AP2/EREBP	1	1	0
P	MYB	2	1	1
smallRNA(fl3)	small RNAs	5	4	1
smallRNA(i)	small RNAs	2	0	2
smallRNA(i2)	small RNAs	4	1	3
smallRNA(le3)	small RNAs	8	0	8
smallRNA(s2)	small RNAs	3	2	1
smallRNA(se3)	small RNAs	3	2	1
smallRNA(si3)	small RNAs	10	4	6
TBP	- other	1	0	1
TEIL	AP2/EREBP	3	3	0
TSS	- other	1	0 (both strands)	
WRKY26	WRKY(Zn)	2	1	1
WRKY38	WRKY(Zn)	1	0	1
WRKY43	WRKY(Zn)	1	0	1
ZmHOX2a(2)	HD-HOX	1	0	1

Note: The Transcription factors which is not presented in table 1b is mark in bold characters.

Supplementary Data 7 The alignment of sAPX and tAPX sequences

The sequences of tAPX (at1g77490.1) and sAPX (at4g08390.1) were retrieved from the Arabidopsis Information Resource. The alignment was down by online tool PRALINE (http://www.ibi.vu.nl/programs/pralinewww/) (Simossis and Heringa, 2005).



6 Reference

Abe, H., YamaguchiShinozaki, K., Urao, T., Iwasaki, T., Hosokawa, D., and Shinozaki, K. (1997). Role of Arabidopsis MYC and MYB homologs in drought- and abscisic acid-regulated gene expression. The Plant Cell **9,** 1859-1868.

Abe, H., Urao, T., Ito, T., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2003). Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. The Plant cell **15,** 63-78.

Agati, G., Matteini, P., Goti, A., and Tattini, M. (2007). Chloroplast-located flavonoids can scavenge singlet oxygen. New Phytologist **174,** 77-89.

Agati, G., Azzarello, E., Pollastri, S., and Tattini, M. (2012). Flavonoids as antioxidants in plants: Location and functional significance. Plant Science **196,** 67-76.

Alfonso, M. (2000). Redox Control of psbA Gene Expression in the Cyanobacterium Synechocystis PCC 6803. Involvement of the Cytochrome b6/f Complex. Plant physiology **122,** 505-516.

Allen, D.J., and Ort, D.R. (2001). Impacts of chilling temperatures on photosynthesis in warm-climate plants. Trends in plant science **6,** 36-42.

Aluru, M.R., Zola, J., Foudree, A., and Rodermel, S.R. (2009). Chloroplast photooxidation-induced transcriptome reprogramming in Arabidopsis immutans white leaf sectors. Plant Physiol **150,** 904-923.

Apel, K., and Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annual Review of Plant Biology **55,** 373-399.

Arase, F., Nishitani, H., Egusa, M., Nishimoto, N., Sakurai, S., Sakamoto, N., and Kaminaka, H. (2012). IAA8 Involved in Lateral Root Formation Interacts with the TIR1 Auxin Receptor and ARF Transcription Factors in Arabidopsis. PLoS One **7**. e43414.

Armbruster, U., Pesaresi, P., Pribil, M., Hertle, A., and Leister, D. (2010). Update on chloroplast research: new tools, new topics, and new trends. Molecular Plant.

Asada, K. (1992). ascorbate peroxidase - a hydrogen peroxide-scavenging enzyme in plants. Physiologia Plantarum **85,** 235-241.

Asada, K. (1999). The water-water cycle in chloroplasts: Scavenging of active oxygens and dissipation of excess photons. Annual Review of Plant Physiology and Plant Molecular Biology **50,** 601-639.

Asada, K. (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiol **141,** 391-396.

Asada, K., Takahashi, M., Tanaka, K., and Nakano, Y. (1977). Formation of active oxygen and its fate in chloroplasts. Biochemical and medical aspects of active oxygen.

Ashton, F.M.C.A.S. (1973). Mode of action of herbicides. (New York: Wiley).

Avraham, R., and Yarden, Y. (2011). Feedback regulation of EGFR signalling: decision making by early and delayed loops. Nature Reviews Molecular Cell Biology **12,** 104-117.

Baier, M., and Dietz, K. (2005). Chloroplasts as source and target of cellular redox regulation: a discussion on chloroplast redox signals in the context of plant physiology. Journal of Experimental Botany **56,** 1449-1462.

Baier, M., Stroher, E., and Dietz, K. (2004). The acceptor availability at photosystem I and ABA control nuclear expression of 2-Cys peroxiredoxin-A in *Arabidopsis thaliana*. Plant Cell Physiol **45,** 997-1006.

Baier, M., Noctor, G., Foyer, C.H., and Dietz, K.J. (2000). Antisense suppression of 2-cysteine peroxiredoxin in Arabidopsis specifically enhances the activities and expression of enzymes associated with ascorbate metabolism but not glutathione metabolism. Plant physiology **124,** 823-832.

Baker, N.R., and Butler, W.L. (1976). Development of the primary photochemical apparatus of photosynthesis during greening of etiolated bean leaves. Plant Physiol **58,** 526-529.

Barb, W.G., Baxendale, J.H., George, P., and Hargrave, K.R. (1951). Reactions of ferrous and ferric ions with hydrogen peroxide. Part II.-The ferric ion reaction. Transactions of the Faraday Society **47,** 591-616.

Barnes, C., Tibbitts, T., Sager, J., Deitzer, G., Bubenheim, D., Koerner, G., and Bugbee, B. (1993). Accuracy of quantum sensors measuring yield photon flux and photosynthetic photon flux. HortScience **28,** 1197-1200.

Baruah, A., Šimková, K., Hincha, D.K., Apel, K., and Laloi, C. (2009). Modulation of 1O2-mediated retrograde signaling by the PLEIOTROPIC RESPONSE LOCUS 1 (PRL1) protein, a central integrator of stress and energy signaling. The Plant Journal **60,** 22-32.

Beck, E., Burkert, A., and Hofmann, M. (1983). Uptake of l-ascorbate by intact spinach chloroplasts. Plant Physiol **73,** 41-45.

Bellafiore, S., Barneche, F., Peltier, G., and Rochaix, J.-D. (2005). State transitions and light adaptation require chloroplast thylakoid protein kinase STN7. Nature **433,** 892-895.

Bernier-Villamor, L., Navarro, E., Sevilla, F., and Lazaro, J.J. (2004). Cloning and characterization of a 2-Cys peroxiredoxin from *Pisum sativum*. Journal of Experimental Botany **55,** 2191-2199.

Biehler, K., and Fock, H. (1996). Evidence for the contribution of the Mehler-peroxidase reaction in dissipating excess electrons in drought-stressed wheat. Plant Physiology **112,** 265-272.

Bienert, G.P., Schjoerring, J.K., and Jahn, T.P. (2006). Membrane transport of hydrogen peroxide. Biochimica et Biophysica Acta (BBA) - Biomembranes **1758,** 994-1003.

Birkenbihl, R.P., Jach, G., Saedler, H., and Huijser, P. (2005). Functional dissection of the plant-specific SBP-domain: Overlap of the DNA-binding and nuclear localization domains. Journal of Molecular Biology **352,** 585-596.

Bradbeer, J.W., Atkinson, Y.E., Borner, T., and Hagemann, R. (1979). Cytoplasmic synthesis of plastid polypeptides may be controlled by plastid-synthesised RNA. Nature **279,** 816-817.

Braybrook, S.A., Stone, S.L., Park, S., Bui, A.Q., Le, B.H., Fischer, R.L., Goldberg, R.B., and Harada, J.J. (2006). Genes directly regulated by LEAFY COTYLEDON2 provide insight into the control of embryo maturation and somatic embryogenesis. Proceedings of the National Academy of Sciences of the United States of America **103,** 3468-3473.

Breitenbach, J., Zhu, C., and Sandmann, G. (2001). Bleaching herbicide norflurazon inhibits phytoene desaturase by competition with the cofactors. Journal of Agricultural and Food Chemistry **49,** 5270-5272.

Bulow, L., Brill, Y., and Hehl, R. (2010). AthaMap-assisted transcription factor target gene identification in *Arabidopsis thaliana*. Database (Oxford) **2010,** baq034.

Bunkelmann, J.R., and Trelease, R.N. (1996). Ascorbate peroxidase - A prominent membrane protein in oilseed glyoxysomes. Plant Physiology **110,** 589-598.

Busk, P.K., and Pages, M. (1998). Regulation of abscisic acid-induced transcription. Plant Molecular Biology. **37,** 425-435.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., and Wittwer, C.T. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry **55,** 611-622.

Cairns, N.G., Pasternak, M., Wachter, A., Cobbett, C.S., and Meyer, A.J. (2006). Maturation of Arabidopsis seeds is dependent on glutathione biosynthesis within the embryo. Plant Physiology **141,** 446-455.

Castillon, A., Shen, H., and Huq, E. (2007). Phytochrome Interacting Factors: central players in phytochrome-mediated light signaling networks. Trends in plant science **12,** 514-521.

Chain, R.K., and Malkin, R. (1979). On the interaction of 2,5-dibromo-3-methyl-6-isopropylbenzoquinone (DBMIB) with bound electron carriers in spinach chloroplasts. Archives of Biochemistry and Biophysics **197,** 52-56.

Chakravarthy, S., Tuori, R.P., D'Ascenzo, M.D., Fobert, P.R., Despres, C., and Martin, G.B. (2003). The tomato transcription factor Pti4 regulates defense-related gene expression via GCC box and non-GCC box cis elements. The Plant Cell **15,** 3033-3050.

Chan, C.S., Guo, L., and Shih, M.C. (2001). Promoter analysis of the nuclear gene encoding the chloroplast glyceraldehyde-3-phosphate dehydrogenase B subunit of *Arabidopsis thaliana*. Plant Molecular Biology **46,** 131-141.

Chaubet, N., Flenet, M., Clement, B., Brignon, P., and Gigot, C. (1996). Identification of *cis*-elements regulating the expression of an Arabidopsis histone H4 gene. The Plant Journal. **10,** 425-435.

Chen, C., and Chen, Z. (2000). Isolation and characterization of two pathogen- and salicylic acid-induced genes encoding WRKY DNA-binding proteins from tobacco. Plant Molecular Biology **42,** 387-396.

Chen, G.X., and Asada, K. (1989). Ascorbate Peroxidase in Tea Leaves - Occurrence of 2 Isozymes and the Differences in Their Enzymatic and Molecular-Properties. Plant & Cell Physiology **30,** 987-998.

Chen, M., and Chory, J. (2011). Phytochrome signaling mechanisms and the control of plant development. Trends in Cell Biology **21,** 664-671.

Chen, W., Provart, N.J., Glazebrook, J., Katagiri, F., Chang, H.-S., Eulgem, T., Mauch, F., Luan, S., Zou, G., Whitham, S.A., Budworth, P.R., Tao, Y., Xie, Z., Chen, X., Lam, S., Kreps, J.A., Harper, J.F., Si-Ammour, A., Mauch-Mani, B., Heinlein, M., Kobayashi, K., Hohn, T., Dangl, J.L., Wang, X., and Zhu, T. (2002). Expression profile matrix of arabidopsis transcription factor genes suggests their putative functions in response to environmental stresses. The Plant Cell **14,** 559-574.

Cheng, N.-H., Liu, J.-Z., Brock, A., Nelson, R.S., and Hirschi, K.D. (2006). AtGRXcp, an Arabidopsis chloroplastic glutaredoxin, is critical for protection against protein oxidative damage. Journal of Biological Chemistry **281,** 26280-26288.

Chew, O., Whelan, J., and Millar, A.H. (2003). Molecular definition of the ascorbate-glutathione cycle in arabidopsis mitochondria reveals dual targeting of antioxidant defenses in plants. Journal of Biological Chemistry **278,** 46869-46877.

Chow, W.S., and Hope, A.B. (1998). The electrochromic signal, redox reactions in the cytochrome bf complex and photosystem functionality in photoinhibited tobacco leaf segments. Australian Journal of Plant Physiology **25,** 775-784.

Clemens, S., Kim, E.J., Neumann, D., and Schroeder, J.I. (1999). Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. Embo Journal **18,** 3325-3333.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method forAgrobacterium-mediated transformation of *Arabidopsis thaliana*. The Plant Journal **16,** 735-743.

Covington, M., Maloof, J., Straume, M., Kay, S., and Harmer, S. (2008). Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. Genome Biology **9,** R130.

Creelman, R.A., Tierney, M.L., and Mullet, J.E. (1992). Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. Proceedings of the National Academy of Sciences of the United States of America **89,** 4938-4941.

Dall'Osto, L., Lico, C., Alric, J., Giuliano, G., Havaux, M., and Bassi, R. (2006). Lutein is needed for efficient chlorophyll triplet quenching in the major LHCII antenna complex of higher plants and effective photoprotection in vivo under strong light. BMC Plant Biology **6**.

Davletova, S., Rizhsky, L., Liang, H., Shengqiang, Z., Oliver, D.J., Coutu, J., Shulaev, V., Schlauch, K., and Mittler, R. (2005). Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of arabidopsis. The Plant Cell **17,** 268-281.

Davuluri, R., Sun, H., Palaniswamy, S., Matthews, N., Molina, C., Kurtz, M., and Grotewold, E. (2003). AGRIS: Arabidopsis Gene Regulatory Information Server, an information resource of Arabidopsis *cis*-regulatory elements and transcription factors. BMC Bioinformatics **4,** 25.

Dehesh, K., Bruce, W.B., and Quail, P.H. (1990). A trans-acting factor that binds to a GT-motif in a phytochrome gene promoter. Science **250,** 1397-1399.

Deng, Z., Oses-Prieto, J.A., Kutschera, U., Tseng, T.-S., Hao, L., Burlingame, A.L., Wang, Z.-Y., and Briggs, W.R. (2014). Blue light-induced proteomic changes in etiolated arabidopsis seedlings. Journal of Proteome Research **13,** 2524-2533.

Devlin, P.F., Yanovsky, M.J., and Kay, S.A. (2003). A genomic analysis of the shade avoidance response in arabidopsis. Plant Physiology **133,** 1617-1629.

Dietz, K., Jacob, S., Oelze, M., Laxa, M., Tognetti, V., de Miranda, S., Baier, M., and Finkemeier, I. (2006). The function of peroxiredoxins in plant organelle redox metabolism. Journal of Experimental Botany **57,** 1697-1709.

Donald, R.G., and Cashmore, A.R. (1990). Mutation of either G box or I box sequences profoundly affects expression from the Arabidopsis rbcS-1A promoter. EMBO Joural. **9,** 1717-1726.

Doulis, A.G., Debian, N., Kingston-Smith, A.H., and Foyer, C.H. (1997). Differential localization of antioxidants in maize leaves. Plant Physiology **114,** 1031-1037.

Dowdle, J., Ishikawa, T., Gatzek, S., Rolinski, S., and Smirnoff, N. (2007). Two genes in *Arabidopsis thaliana* encoding GDP-L-galactose phosphorylase are required for ascorbate biosynthesis and seedling viability. The Plant Journal **52,** 673-689.

Dunford, H.B., and Stillman, J.S. (1976). On the function and mechanism of action of peroxidases. Coordination Chemistry Reviews **19,** 187-251.

Durner, J., and Klessig, D.F. (1995). Inhibition of ascorbate peroxidase by salicylic acid and 2,6-dichloroisonicotinic acid, two inducers of plant defense responses. Proceedings of the National Academy of Sciences of the United States of America **92,** 11312-11316.

Ellis, R.J. (1975). Protein-synthesis in chloroplasts .3. inhibition of chloroplast protein-synthesis by lincomycin and 2-(4-methyl-2,6-dinitroanilino)-n-methylpropionamide. Phytochemistry **14,** 89-93.

Erdei, N., Barta, C., Hideg, E., and Boddi, B. (2005). Light-induced wilting and its molecular mechanism in epicotyls of dark-germinated pea (*Pisum sativum* L.) seedlings. Plant Cell Physiology **46,** 185-191.

Estavillo, G.M., Crisp, P.A., Pornsiriwong, W., Wirtz, M., Collinge, D., Carrie, C., Giraud, E., Whelan, J., David, P., Javot, H., Brearley, C., Hell, R., Marin, E., and Pogson, B.J. (2011). Evidence for a SAL1-PAP chloroplast retrograde pathway that functions in drought and high light signaling in Arabidopsis. The Plant Cell **23,** 3992-4012.

Flexas, J., and Medrano, H. (2002). Drought-inhibition of Photosynthesis in C3 Plants: Stomatal and Non-stomatal Limitations Revisited. Annals of Botany **89,** 183-189.

Foyer, C., and Halliwell, B. (1976). Presence of glutathione and glutathione reductase in chloroplasts - proposed role in ascorbic-acid metabolism. Planta **133,** 21-25.

Foyer, C., Rowell, J., and Walker, D. (1983). Measurement of the ascorbate content of spinach leaf protoplasts and chloroplasts during illumination. Planta **157,** 239-244.

Foyer, C.H., and Noctor, G. (2000). Tansley review no. 112. New Phytologist **146,** 359-388.

Foyer, C.H., and Noctor, G. (2009). Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. Antioxidants & Redox Signaling **11,** 861-905.

Foyer, C.H., and Noctor, G. (2011). Ascorbate and glutathione: the heart of the redox hub. Plant Physiology **155,** 2-18.

Foyer, C.H., Bloom, A.J., Queval, G., and Noctor, G. (2009). Photorespiratory metabolism: genes, mutants, energetics, and redox signaling. Annual Review of Plant Biology **60,** 455-484.

Fridovich, I. (1978). The biology of oxygen radicals. Science **201,** 875-880.

Friso, G., Majeran, W., Huang, M., Sun, Q., and van Wijk, K.J. (2010). Reconstruction of metabolic pathways, protein expression, and homeostasis machineries across maize bundle sheath and mesophyll chloroplasts: large-scale quantitative proteomics using the first maize genome assembly. Plant Physiology **152,** 1219-1250.

Fryer, M.J., Ball, L., Oxborough, K., Karpinski, S., Mullineaux, P.M., and Baker, N.R. (2003). Control of Ascorbate Peroxidase 2 expression by hydrogen peroxide and leaf water status during excess light stress reveals a functional organisation of Arabidopsis leaves. The Plant Journal **33,** 691-705.

Gabrielsen, E.K., Madsen, A., and Vejlby, K. (1961). Induction of photosynthesis in etiolated leaves. Physiologia Plantarum **14,** 98-110.

Gadea, J., Conejero, V., and Vera, P. (1999). Developmental regulation of a cytosolic ascorbate peroxidase gene from tomato plants. Molecular and General Genetics **262,** 212-219.

Gallagher, S.R. (1992). GUS protocols : using the GUS gene as a reporter of gene expression. (San Diego: Academic Press).

Gallie, D.R. (2013). L-ascorbic Acid: a multifunctional molecule supporting plant growth and development. Scientifica (Cairo) **2013,** 795964.

Galvez-Valdivieso, G., Fryer, M., Lawson, T., Slattery, K., Truman, W., Smirnoff, N., Asami, T., Davies, W., Jones, A., Baker, N., and Mullineaux, P. (2009). The high light response in Arabidopsis involves ABA signaling between vascular and bundle sheath cells. The Plant Cell **21,** 2143-2162.

Gama, F., Brehelin, C., Gelhaye, E., Meyer, Y., Jacquot, J.P., Rey, P., and Rouhier, N. (2008). Functional analysis and expression characteristics of chloroplastic Prx IIE. Physiologia Plantarum **133,** 599-610.

Gavazzi, F., Lazzari, B., Ciceri, P., Gianazza, E., and Viotti, A. (2007). Wild-type Opaque2 and defective opaque2 polypeptides form complexes in maize endosperm cells and bind the Opaque2-Zein target site. Plant physiology **145,** 933-945.

Gerhardt, B. (1964). Untersuchungen über Beziehungen zwischen Ascorbinsäure und Photosynthese. Planta **61,** 101-129.

Giacomelli, L., Masi, A., Ripoll, D.R., Lee, M.J., and van Wijk, K.J. (2007). *Arabidopsis thaliana* deficient in two chloroplast ascorbate peroxidases shows accelerated light-induced necrosis when levels of cellular ascorbate are low. Plant Molecular Biology **65,** 627-644.

Gläßer, C., Haberer, G., Finkemeier, I., Pfannschmidt, T., Kleine, T., Leister, D., Dietz, K.-J., Häusler, R.E., Grimm, B., and Mayer, K.F.X. (2014). Meta-analysis of retrograde signaling in *Arabidopsis thaliana* reveals a core module of genes embedded in complex cellular signaling networks. Molecular Plant **7,** 1167-90.

Goda, H., Sawa, S., Asami, T., Fujioka, S., Shimada, Y., and Yoshida, S. (2004). Comprehensive comparison of auxin-regulated and brassinosteroid-regulated genes in Arabidopsis. Plant Physiology. **134,** 1555-1573.

Golding, A.J., Finazzi, G., and Johnson, G.N. (2004). Reduction of the thylakoid electron transport chain by stromal reductants-evidence for activation of cyclic electron transport upon dark adaptation or under drought. Planta **220,** 356-363.

Gorman, A.A., and Rodgers, M.A. (1992). Current perspectives of singlet oxygen detection in biological environments. Journal of photochemistry and photobiology. B, Biology **14,** 159-176.

Gotz, T., Sandmann, G., and Romer, S. (2002). Expression of a bacterial carotene hydroxylase gene (crtZ) enhances UV tolerance in tobacco. Plant Molecular Biology **50,** 129-142.

Goyer, A., Haslekås, C., Miginiac-Maslow, M., Klein, U., Le Marechal, P., Jacquot, J.-P., and Decottignies, P. (2002). Isolation and characterization of a thioredoxin-dependent peroxidase from *Chlamydomonas reinhardtii*. European Journal of Biochemistry **269,** 272-282.

Granlund, I., Storm, P., Schubert, M., Garcia-Cerdan, J.G., Funk, C., and Schroder, W.P. (2009). The TL29 protein is lumen located, associated with PSII and not an ascorbate peroxidase. Plant Cell Physiology **50,** 1898-1910.

Grant, J.J., Yun, B.W., and Loake, G.J. (2000). Oxidative burst and cognate redox signalling reported by luciferase imaging: identification of a signal network that functions independently of ethylene, SA and Me-JA but is dependent on MAPKK activity. The Plant Journal **24,** 569-582.

Green, P.J., Kay, S.A., and Chua, N.H. (1987). Sequence-specific interactions of a pea nuclear factor with light-responsive elements upstream of the rbcS-3A gene. EMBO Journal. **6,** 2543-2549.

Griffiths, W.T. (1978). Reconstitution of chlorophyllide formation by isolated etioplast membranes. Biochemical Journal **174,** 681-692.

Grill, E., Winnacker, E.L., and Zenk, M.H. (1987). Phytochelatins, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins. Proceedings of the National Academy of Sciences of the United States of America **84,** 439-443.

Groden, D., and Beck, E. (1979). H_2O_2 destruction by ascorbate-dependent systems from chloroplasts. Biochimica et Biophysica Acta **546,** 426-435.

Groen, A., Lemeer, S., van der Wijk, T., Overvoorde, J., Heck, A.J., Ostman, A., Barford, D., Slijper, M., and den Hertog, J. (2005). Differential oxidation of protein-tyrosine phosphatases. Journal of Biological Chemistry **280,** 10298-10304.

Ha, S.B., Smith, A.P., Howden, R., Dietrich, W.M., Bugg, S., O'Connell, M.J., Goldsbrough, P.B., and Cobbett, C.S. (1999). Phytochelatin synthase genes from arabidopsis and the yeast Schizosaccharomyces pombe. The Plant Cell **11,** 1153-1163.

Haber, F., and Weiss, J. (1932). Über die Katalyse des Hydroperoxydes. Naturwissenschaften **20,** 948-950.

Hahn, D., Kaltenbach, C., and Kuck, U. (1998). The Calvin cycle enzyme sedoheptulose-1,7-bisphosphatase is encoded by a light-regulated gene in *Chlamydomonas reinhardtii*. Plant Molecular Biology **36,** 929-934.

Halliwell, B., and Gutteridge, J.M. (1985). Free radicals in biology and medicine. (Oxford: Clarendon Pr.).

Hass, C., Lohrmann, J., Albrecht, V., Sweere, U., Hummel, F., Yoo, S.D., Hwang, I., Zhu, T., Schafer, E., Kudla, J., and Harter, K. (2004). The response regulator 2 mediates ethylene signalling and hormone signal integration in Arabidopsis. EMBO Journal **23,** 3290-3302.

Hatzios, K.K., Penner, D., and Bell, D. (1980). Inhibition of photosynthetic electron transport in isolated spinach chloroplasts by two 1,3,4-thiadiazolyl derivatives. Plant Physiology **65,** 319-321.

Havaux, M. (1998). Carotenoids as membrane stabilizers in chloroplasts. Trends in Plant Science **3,** 147-151.

Havaux, M., and Niyogi, K.K. (1999). The violaxanthin cycle protects plants from photooxidative damage by more than one mechanism. Proceedings of the National Academy of Sciences of the United States of America **96,** 8762-8767.

Havaux, M., Dall'Osto, L., and Bassi, R. (2007). Zeaxanthin has enhanced antioxidant capacity with respect to all other xanthophylls in arabidopsis leaves and functions independent of binding to psii antennae. Plant Physiology **145,** 1506-1520.

Havaux, M., Eymery, F., Porfirova, S., Rey, P., and Dormann, P. (2005). Vitamin E protects against photoinhibition and photooxidative stress in *Arabidopsis thaliana*. The Plant Cell **17,** 3451-3469.

Hebbelmann, I., Selinski, J., Wehmeyer, C., Goss, T., Voss, I., Mulo, P., Kangasjarvi, S., Aro, E.M., Oelze, M.L., Dietz, K.J., Nunes-Nesi, A., Do, P.T., Fernie, A.R., Talla, S.K., Raghavendra, A.S., Linke, V., and Scheibe, R. (2012). Multiple strategies to prevent oxidative stress in Arabidopsis plants lacking the malate valve enzyme NADP-malate dehydrogenase. Journal of Experimental Botany **63,** 1445-1459.

Hehl, R., and Bulow, L. (2014). AthaMap web tools for the analysis of transcriptional and posttranscriptional regulation of gene expression in *Arabidopsis thaliana*. Methods Plant Molecular Biology **1158,** 139-156.

Heiber, I., Cai, W., and Baier, M. (2014). Linking chloroplast antioxidant defense to carbohydrate availability: the transcript abundance of stromal ascorbate peroxidase is sugar-controlled via ascorbate biosynthesis. Molecular Plant **7,** 58-70.

Heiber, I., Stroher, E., Raatz, B., Busse, I., Kahmann, U., Bevan, M., Dietz, K., and Baier, M. (2007a). The redox imbalanced mutants of arabidopsis differentiate signaling pathways for redox regulation of chloroplast antioxidant enzymes. Plant Physiology. **143,** 1774-1788.

Henrissat, B., Saloheimo, M., Lavaitte, S., and Knowles, J.K. (1990). Structural homology among the peroxidase enzyme family revealed by hydrophobic cluster analysis. Proteins **8,** 251-257.

Higo, K., Ugawa, Y., Iwamoto, M., and Korenaga, T. (1999). Plant *cis*-acting regulatory DNA elements (PLACE) database: 1999. Nucleic Acids Research **27,** 297-300.

Holmgren, A. (1989). Thioredoxin and glutaredoxin systems. Journal of Biological Chemistry **264,** 13963-13966.

Holt, N.E., Fleming, G.R., and Niyogi, K.K. (2004). Toward an understanding of the mechanism of nonphotochemical quenching in green plants. Biochemistry **43,** 8281-8289.

Hong, C.Y., and Kao, C.H. (2008). NaCl-induced expression of ASCORBATE PEROXIDASE 8 in roots of rice (*Oryza sativa* L.) seedlings is not associated with osmotic component. Plant Signal Behav **3,** 199-201.

Horemans, N., Foyer, C.H., Potters, G., and Asard, H. (2000). Ascorbate function and associated transport systems in plants. Plant Physiology and Biochemistry **38,** 531-540.

Horling, F., Lamkemeyer, P., Konig, J., Finkemeier, I., Kandlbinder, A., Baier, M., and Dietz, K. (2003). Divergent light-, ascorbate-, and oxidative stress-dependent regulation of expression of the peroxiredoxin gene family in Arabidopsis. Plant Physiology **131,** 317-325.

Hosoda, K., Imamura, A., Katoh, E., Hatta, T., Tachiki, M., Yamada, H., Mizuno, T., and Yamazaki, T. (2002). Molecular structure of the GARP family of plant Myb-related DNA binding motifs of the arabidopsis response regulators. The Plant Cell **14,** 2015-2029.

Hossain, M.A., and Asada, K. (1984). Inactivation of ascorbate peroxidase in spinach chloroplasts on dark addition of hydrogen peroxide: its protection by ascorbate. Plant & Cell Physiology **25,** 1285-1295.

Hu, Y.-Q., Liu, S., Yuan, H.-M., Li, J., Yan, D.-W., Zhang, J.-F., and Lu, Y.-T. (2010). Functional comparison of catalase genes in the elimination of photorespiratory H_2O_2 using promoter- and 3' -untranslated region exchange experiments in the Arabidopsis *cat2* photorespiratory mutant. Plant, cell & environment **33,** 1656-1670.

Hu, Y.X., Wang, Y.H., Liu, X.F., and Li, J.Y. (2004). Arabidopsis RAV1 is down-regulated by brassinosteroid and may act as a negative regulator during plant development. Cell Research **14,** 8-15.

Hudson, M.E., and Quail, P.H. (2003). Identification of promoter motifs involved in the network of phytochrome A-regulated gene expression by combined analysis of genomic sequence and microarray data. Plant Physiology. **133,** 1605-1616.

Hung, K.T., Hsu, Y.T., and Kao, C.H. (2006). Hydrogen peroxide is involved in methyl jasmonate-induced senescence of rice leaves. Physiologia Plantarum **127,** 293-303.

Iqbal, A., Yabuta, Y., Takeda, T., Nakano, Y., and Shigeoka, S. (2006). Hydroperoxide reduction by thioredoxin-specific glutathione peroxidase isoenzymes of *Arabidopsis thaliana*. FEBS Journal **273,** 5589-5597.

Ishida, K., Yamashino, T., Yokoyama, A., and Mizuno, T. (2008). Three type-B response regulators, ARR1, ARR10 and ARR12, play essential but redundant roles in cytokinin signal transduction throughout the life cycle of *Arabidopsis thaliana*. Plant Cell Physiology **49,** 47-57.

Iwasaki, T., Yamaguchishinozaki, K., and Shinozaki, K. (1995). Identification of a *cis*-regulatory region of a gene in *Arabidopsis thaliana* whose induction by dehydration is mediated by abscisic-acid and requires protein-synthesis. Molecular and General Genetics **247,** 391-398.

Jakoby, M.J., Falkenhan, D., Mader, M.T., Brininstool, G., Wischnitzki, E., Platz, N., Hudson, A., Hulskamp, M., Larkin, J., and Schnittger, A. (2008). Transcriptional profiling of mature Arabidopsis trichomes reveals that NOECK encodes the MIXTA-like transcriptional regulator MYB106. Plant physiology **148,** 1583-1602.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO Journal **6,** 3901-3907.

Jimenez, A., Hernandez, J.A., delRio, L.A., and Sevilla, F. (1997). Evidence for the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea leaves. Plant Physiology **114,** 275-284.

Johnson, M.P., Havaux, M., Triantaphylides, C., Ksas, B., Pascal, A.A., Robert, B., Davison, P.A., Ruban, A.V., and Horton, P. (2007). Elevated zeaxanthin bound to oligomeric LHCII enhances the resistance of Arabidopsis to photooxidative stress by a lipid-protective, antioxidant mechanism. Journal of Biological Chemistry **282**.

Joo, J.H., Bae, Y.S., and Lee, J.S. (2001). Role of auxin-induced reactive oxygen species in root gravitropism. Plant Physiology **126,** 1055-1060.

Juszczak, I. (2013). Natural genetic variation in the expression regulation of chloroplast antioxidant system among *A. thaliana* accessions (Berlin: Freie Universität Berlin).

Juszczak, I., Rudnik, R., Pietzenuk, B., and Baier, M. (2012). Natural genetic variation in the expression regulation of the chloroplast antioxidant system among *Arabidopsis thaliana* accessions. Physiologia Plantarum **146,** 53-70.

Kagawa, T., Sakai, T., Suetsugu, N., Oikawa, K., Ishiguro, S., Kato, T., Tabata, S., Okada, K., and Wada, M. (2001). Arabidopsis NPL1: a phototropin homolog controlling the chloroplast high-light avoidance response. Science **291,** 2138-2141.

Kagaya, Y., Ohmiya, K., and Hattori, T. (1999). RAV1, a novel DNA-binding protein, binds to bipartite recognition sequence through two distinct DNA-binding domains uniquely found in higher plants. Nucleic Acids Research . **27,** 470-478.

Kaiser, W.M. (1979). Reversible inhibition of the calvin cycle and activation of oxidative pentose-phosphate cycle in isolated intact chloroplasts by hydrogen-peroxide. Planta **145,** 377-382.

Kalachova, T., Iakovenko, O., Kretinin, S., and Kravets, V. (2013). Involvement of phospholipase D and NADPH-oxidase in salicylic acid signaling cascade. Plant Physiology and Biochemistry **66,** 127-133.

Kangasjärvi, S., Lepisto, A., Hannikainen, K., Piippo, M., Luomala, E.M., Aro, E.M., and Rintamaki, E. (2008). Diverse roles for chloroplast stromal and thylakoid-bound ascorbate peroxidases in plant stress responses. Biochemical Journal **412,** 275-285.

Kanwischer, M., Porfirova, S., Bergmuller, E., and Dormann, P. (2005). Alterations in tocopherol cyclase activity in transgenic and mutant plants of Arabidopsis affect tocopherol content, tocopherol composition, and oxidative stress. Plant Physiol **137,** 713-723.

Karimi, M., Inze, D., and Depicker, A. (2002). GATEWAY vectors for Agrobacterium-mediated plant transformation. Trends Plant Science **7,** 193-195.

Keeling, P.J. (2010). The endosymbiotic origin, diversification and fate of plastids. Philosophical Transactions of the Royal Society B: Biological Sciences **365,** 729-748.

Keren, N., Berg, A., Van Kan, P.J., Levanon, H., and Ohad, I. (1997). Mechanism of photosystem II photoinactivation and D1 protein degradation at low light: the role of back electron flow. Proceedings of the National Academy of Sciences of the United States of America **94,** 1579-1584.

Kiddle, G., Pastori, G.M., Bernard, S., Pignocchi, C., Antoniw, J., Verrier, P.J., and Foyer, C.H. (2003a). Effects of leaf ascorbate content on defense and photosynthesis gene expression in *Arabidopsis thaliana*. Antioxidants & Redox Signaling. **5,** 23-32.

Kiddle, G., Pastori, G.M., Bernard, S., Pignocchi, C., Antoniw, J., Verrier, P.J., and Foyer, C.H. (2003b). Effects of leaf ascorbate content on defense and photosynthesis gene expression in *Arabidopsis thaliana*. Antioxidants & Redox Signaling **5,** 23-32.

Kieselbach, T., Bystedt, M., Hynds, P., Robinson, C., and Schroder, W.P. (2000). A peroxidase homologue and novel plastocyanin located by proteomics to the Arabidopsis chloroplast thylakoid lumen. FEBS Letters **480,** 271-276.

Kim, J., Rudella, A., Ramirez Rodriguez, V., Zybailov, B., Olinares, P.D., and van Wijk, K.J. (2009). Subunits of the plastid ClpPR protease complex have differential contributions to embryogenesis, plastid biogenesis, and plant development in Arabidopsis. The Plant cell **21,** 1669-1692.

Kimura, M., Yamamoto, Y.Y., Seki, M., Sakurai, T., Sato, M., Abe, T., Yoshida, S., Manabe, K., Shinozaki, K., and Matsui, M. (2003). Identification of Arabidopsis genes regulated by high light-stress using cDNA microarray. Photochemistry and Photobiology **77,** 226-233.

Kindgren, P., Kremnev, D., Blanco, N.E., de Dios Barajas Lopez, J., Fernandez, A.P., Tellgren-Roth, C., Kleine, T., Small, I., and Strand, A. (2012). The plastid redox insensitive 2 mutant of Arabidopsis is impaired in PEP activity and high light-dependent plastid redox signalling to the nucleus. The Plant Journal **70,** 279-291.

Kinsman, E.A., and Pyke, K.A. (1998). Bundle sheath cells and cell-specific plastid development in Arabidopsis leaves. Development **125,** 1815-1822.

Kitajima, S. (2008). Hydrogen peroxide-mediated inactivation of two chloroplastic peroxidases, ascorbate peroxidase and 2-cys peroxiredoxin. Photochemistry and Photobiology **84,** 1404-1409.

Kitajima, S., Tomizawa, K., Shigeoka, S., and Yokota, A. (2006). An inserted loop region of stromal ascorbate peroxidase is involved in its hydrogen peroxide-mediated inactivation. FEBS Journal **273,** 2704-2710.

Klein, P., Seidel, T., Stocker, B., and Dietz, K.J. (2012). The membrane-tethered transcription factor ANAC089 serves as redox-dependent suppressor of stromal ascorbate peroxidase gene expression. Frontiers in Plant Science **3,** 247.

Knox, J.P., and Dodge, A.D. (1985). Singlet oxygen and plants. Phytochemistry **24,** 889-896.

Koncz, C., and Schell, J. (1986). The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector. Molecular and General Genetics MGG **204,** 383-396.

König, J., Galliardt, H., Jutte, P., Schaper, S., Dittmann, L., and Dietz, K.J. (2013). The conformational bases for the two functionalities of 2-cysteine peroxiredoxins as peroxidase and chaperone. Journal of Experimental Botany **64,** 3483-3497.

Kornyeyev, D., Logan, B.A., Allen, R.D., and Holaday, A.S. (2003). Effect of chloroplastic overproduction of ascorbate peroxidase on photosynthesis and photoprotection in cotton leaves subjected to low temperature photoinhibition. Plant Science **165,** 1033-1041.

Koussevitzky, S., Nott, A., Mockler, T., Hong, F., Sachetto-Martins, G., Surpin, M., Lim, J., Mittler, R., and Chory, J. (2007). Signals from chloroplasts converge to regulate nuclear gene expression. Science **316,** 715-719.

Kreslavski, V.D., Shirshikova, G.N., Lyubimov, V.Y., Shmarev, A.N., Boutanaev, A.M., Kosobryukhov, A.A., Schmitt, F.-J., Friedrich, T., and Allakhverdiev, S.I. (2013). Effect of preillumination with red light on photosynthetic parameters and oxidant-/antioxidant balance in *Arabidopsis thaliana* in response to UV-A. Journal of Photochemistry and Photobiology B: Biology **127,** 229-236.

Krieger-Liszkay, A. (2005). Singlet oxygen production in photosynthesis. Journal of Experimental Botany **56,** 337-346.

Krieger-Liszkay, A., Fufezan, C., and Trebst, A. (2008). Singlet oxygen production in photosystem II and related protection mechanism. Photosynthesis Research **98,** 551-564.

Kristensen, B.K., Askerlund, P., Bykova, N.V., Egsgaard, H., and Møller, I.M. (2004). Identification of oxidised proteins in the matrix of rice leaf mitochondria by immunoprecipitation and two-dimensional liquid chromatography-tandem mass spectrometry. Phytochemistry **65,** 1839-1851.

Kruk, J., Hollander-Czytko, H., Oettmeier, W., and Trebst, A. (2005). Tocopherol as singlet oxygen scavenger in photosystem II. Journal of Plant Physiology **162,** 749-757.

Krusell, L., Rasmussen, I., and Gausing, K. (1997). DNA binding sites recognised *in vitro* by a knotted class 1 homeodomain protein encoded by the hooded gene, k, in barley (*Hordeum vulgare*). FEBS Letters **408,** 25-29.

Kumar, G.N., Iyer, S., and Knowles, N.R. (2007). Strboh A homologue of NADPH oxidase regulates wound-induced oxidative burst and facilitates wound-healing in potato tubers. Planta **227,** 25-36.

Kuno, N., Muramatsu, T., Hamazato, F., and Furuya, M. (2000). Identification by large-scale screening of phytochrome-regulated genes in etiolated seedlings of Arabidopsis using a fluorescent differential display technique. Plant Physiology **122,** 15-24.

Kushnir, S., Babiychuk, E., Kampfenkel, K., Bellesboix, E., Vanmontagu, M., and Inze, D. (1995). Characterization of *Arabidopsis thaliana* cDNA that render yeasts tolerant toward the thiol-oxidizing drug diamide. Proceedings of the National Academy of Sciences of the United States of America **92,** 10580-10584.

Kwak, J.M., Mori, I.C., Pei, Z.M., Leonhardt, N., Torres, M.A., Dangl, J.L., Bloom, R.E., Bodde, S., Jones, J.D., and Schroeder, J.I. (2003). NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in Arabidopsis. EMBO Journal **22,** 2623-2633.

Laloi, C., Mestres-Ortega, D., Marco, Y., Meyer, Y., and Reichheld, J.P. (2004). The Arabidopsis cytosolic thioredoxin *h5* gene induction by oxidative stress and its W-box-mediated response to pathogen elicitor. Plant Physiology. **134,** 1006-1016.

Laloi, C., Stachowiak, M., Pers-Kamczyc, E., Warzych, E., Murgia, I., and Apel, K. (2007). Cross-talk between singlet oxygen- and hydrogen peroxide-dependent signaling of stress responses in *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences **104,** 672-677.

Law, M.Y., Charles, S.A., and Halliwell, B. (1983). Glutathione and ascorbic-acid in spinach (*Spinacia Oleracea*) chloroplasts - the effect of hydrogen-peroxide and of paraquat. Biochemical Journal **210,** 899-903.

Lawlor, D.W., and Cornic, G. (2002). Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant Cell & Environment **25,** 275-294.

Lee, J., He, K., Stolc, V., Lee, H., Figueroa, P., Gao, Y., Tongprasit, W., Zhao, H., Lee, I., and Deng, X.W. (2007a). Analysis of transcription factor HY5 genomic binding sites revealed its hierarchical role in light regulation of development. The Plant Cell **19,** 731-749.

Lee, K., Kim, C., Landgraf, F., and Apel, K. (2007b). EXECUTER1- and EXECUTER2-dependent transfer of stress-related signals from the plastid to the nucleus of *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences of the United States of America **104,** 10270-10275.

Lee, Y., Rubio, M.C., Alassimone, J., and Geldner, N. (2013). A mechanism for localized lignin deposition in the endodermis. Cell **153,** 402-412.

Leister, D., Romani, I., Mittermayr, L., Paieri, F., Fenino, E., and Kleine, T. (2014). Identification of target genes and transcription factors implicated in translation-dependent retrograde signaling in arabidopsis. Molecular Plant **7,** 1228-1247.

Leivar, P., Tepperman, J.M., Monte, E., Calderon, R.H., Liu, T.L., and Quail, P.H. (2009). Definition of early transcriptional circuitry involved in light-induced reversal of PIF-imposed repression of photomorphogenesis in young Arabidopsis seedlings. The Plant Cell **21,** 3535-3553.

Leon, J. (2001). Wound signalling in plants. Journal of Experimental Botany **52,** 1-9.

Leustek, T., and Saito, K. (1999). Sulfate transport and assimilation in plants. Plant Physiology **120,** 637-643.

Lichtenthaler, H.K., Buschmann, C., Döll, M., Fietz, H.J., Bach, T., Kozel, U., Meier, D., and Rahmsdorf, U. (1981). Photosynthetic activity, chloroplast ultrastructure, and leaf characteristics of high-light and low-light plants and of sun and shade leaves. Photosynthesis Research **2,** 115-141.

Life Technologies. pCR8/GW/TOPO Vector Map (Life Technologies).

Lin, C. (2002). Blue light receptors and signal transduction. The Plant Cell **14 Suppl,** S207-225.

Lin, C., and Thomashow, M.F. (1992). DNA sequence analysis of a complementary DNA for cold-regulated Arabidopsis gene *Cor15* and Characterization of the COR 15 polypeptide. Plant physiology **99,** 519-525.

Lin, K.H., and Pu, S.F. (2010). Tissue- and genotype-specific ascorbate peroxidase expression in sweet potato in response to salt stress. Biologia Plantarum **54,** 664-670.

Lindermayr, C., Sell, S., Muller, B., Leister, D., and Durner, J. (2010). Redox regulation of the NPR1-TGA1 system of *Arabidopsis thaliana* by nitric oxide. The Plant cell **22,** 2894-2907.

Liu, K.L., Shen, L., Wang, J.Q., and Sheng, J.P. (2008). Rapid inactivation of chloroplastic ascorbate peroxidase is responsible for oxidative modification to Rubisco in tomato (*Lycopersicon esculentum*) under cadmium stress. Journal of Integrative Plant Biology **50,** 415-426.

Liu, Y., Ren, D., Pike, S., Pallardy, S., Gassmann, W., and Zhang, S. (2007). Chloroplast-generated reactive oxygen species are involved in hypersensitive response-like cell death mediated by a mitogen-activated protein kinase cascade. The Plant Journal **51,** 941-954.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods (San Diego, Calif.) **25,** 402-408.

Lohmann, J.U., Hong, R.L., Hobe, M., Busch, M.A., Parcy, F., Simon, R., and Weigel, D. A molecular link between stem cell regulation and floral patterning in arabidopsis. Cell **105,** 793-803.

Lohrmann, J., Sweere, U., Zabaleta, E., Baurle, I., Keitel, C., Kozma-Bognar, L., Brennicke, A., Schafer, E., Kudla, J., and Harter, K. (2001). The response regulator ARR2: a pollen-specific transcription factor involved in the expression of nuclear genes for components of mitochondrial complex I in Arabidopsis. Molecular Genetics and Genomics **265,** 2-13.

Lovelock, C.E., and Winter, K. (1996). Oxygen-dependent electron transport and protection from photoinhibition in leaves of tropical tree species. Planta **198,** 580-587.

Ma, L., Zhao, H., and Deng, X.W. (2003). Analysis of the mutational effects of the COP/DET/FUS loci on genome expression profiles reveals their overlapping yet not identical roles in regulating Arabidopsis seedling development. Development **130,** 969-981.

Madhusudhan, R., Ishikawa, T., Sawa, Y., Shigeoka, S., and Shibata, H. (2003). Characterization of an ascorbate peroxidase in plastids of tobacco BY-2 cells. Physiologia Plantarum **117,** 550-557.

Majeran, W., Cai, Y., Sun, Q., and van Wijk, K.J. (2005). Functional differentiation of bundle sheath and mesophyll maize chloroplasts determined by comparative proteomics. The Plant Cell **17,** 3111-3140.

Mano, J., Hideg, E., and Asada, K. (2004). Ascorbate in thylakoid lumen functions as an alternative electron donor to photosystem II and photosystem I. Archives of Biochemistry and Biophysics **429,** 71-80.

Mano, J.i., Ohno, C., Domae, Y., and Asada, K. (2001). Chloroplastic ascorbate peroxidase is the primary target of methylviologen-induced photooxidative stress in spinach

leaves: its relevance to monodehydroascorbate radical detected with *in vivo* ESR. Biochimica et Biophysica Acta (BBA) - Bioenergetics **1504,** 275-287.

Mano, S., Yamaguchi, K., Hayashi, M., and Nishimura, M. (1997). Stromal and thylakoid-bound ascorbate peroxidases are produced by alternative splicing in pumpkin. FEBS Letters **413,** 21-26.

Maruta, T., Tanouchi, A., Tamoi, M., Yabuta, Y., Yoshimura, K., Ishikawa, T., and Shigeoka, S. (2010). Arabidopsis chloroplastic ascorbate peroxidase isoenzymes play a dual role in photoprotection and gene regulation under photooxidative stress. Plant & Cell Physiology **51,** 190-200.

Maruta, T., Noshi, M., Tanouchi, A., Tamoi, M., Yabuta, Y., Yoshimura, K., Ishikawa, T., and Shigeoka, S. (2012). H_2O_2-triggered retrograde signaling from chloroplasts to nucleus plays specific role in response to stress. The Journal of Biological Chemistry **287,** 11717-11729.

May, M.J., and Leaver, C.J. (1993). Oxidative stimulation of glutathione synthesis in arabidopsis thaliana suspension-cultures. Plant Physiology **103,** 621-627.

Mayfield, S.P., Yohn, C.B., Cohen, A., and Danon, A. (1995). Regulation of chloroplast gene expression. Annual Review of Plant Physiology and Plant Molecular Biology **46,** 147-166.

Mazzella, M.A., Arana, M.V., Staneloni, R.J., Perelman, S., Rodriguez Batiller, M.J., Muschietti, J., Cerdan, P.D., Chen, K., Sanchez, R.A., Zhu, T., Chory, J., and Casal, J.J. (2005). Phytochrome control of the Arabidopsis transcriptome anticipates seedling exposure to light. The Plant Cell **17,** 2507-2516.

Medina, J.n., Bargues, M., Terol, J., Pérez-Alonso, M., and Salinas, J. (1999). The Arabidopsis CBF gene family is composed of three genes encoding AP2 domain-containing proteins whose expression is regulated by low temperature but not by abscisic acid or dehydration. Plant physiology **119,** 463-470.

Mehler, A.H. (1951). Studies on reactions of illuminated chloroplasts: I. Mechanism of the reduction of oxygen and other hill reagents. Archives of Biochemistry and Biophysics **33,** 65-77.

Meister, A. (1995). Glutathione biosynthesis and its inhibition. Biothiols, Pt B **252,** 26-30.

Mellenthin, M., Ellersiek, U., Börger, A., and Baier, M. (2014). Expression of the Arabidopsis sigma factor *Sig5* is photoreceptor and photosynthesis controlled. Plants **3,** 359-391.

Mellenthin, M. (2012). Identification and characterization of light-responsive *cis*-acting elements in nuclear promoters of *Arabidopsis thaliana* (Düsseldorf: Universitäts- und Landesbibliothek der Heinrich-Heine-Universität Düsseldorf).

Meneguzzo, S., Sgherri, C.L.M., Navari-Izzo, F., and Izzo, R. (1998). Stromal and thylakoid-bound ascorbate peroxidases in NaCl-treated wheat. Physiologia Plantarum **104,** 735-740.

Meyer, A.J., and Fricker, M.D. (2002). Control of demand-driven biosynthesis of glutathione in green Arabidopsis suspension culture cells. Plant Physiology **130,** 1927-1937.

Miller, G., Suzuki, N., Rizhsky, L., Hegie, A., Koussevitzky, S., and Mittler, R. (2007). Double mutants deficient in cytosolic and thylakoid ascorbate peroxidase reveal a complex mode of interaction between reactive oxygen species, plant development, and response to abiotic stresses. Plant Physiology **144,** 1777-1785.

Mittler, R., Vanderauwera, S., Gollery, M., and Van Breusegem, F. (2004). Reactive oxygen gene network of plants. Trends Plant Science **9,** 490-498.

Miyake, C. (2010). Alternative electron flows (water-water cycle and cyclic electron flow around PSI) in photosynthesis: molecular mechanisms and physiological Functions. Plant & Cell Physiology **51,** 1951-1963.

Miyake, C., and Asada, K. (1992). Thylakoid-bound ascorbate peroxidase in spinach-chloroplasts and photoreduction of its primary oxidation-product monodehydroascorbate radicals in thylakoids. Plant & Cell Physiology **33,** 541-553.

Miyake, C., and Asada, K. (1996). Inactivation mechanism of ascorbate peroxidase at low concentrations of ascorbate: Hydrogen peroxide decomposes compound I of ascorbate peroxidase. Plant Cell Physiology **37,** 423-430.

Miyake, C., Cao, W.H., and Asada, K. (1992). Molecular-properties of thylakoid bound ascorbate peroxidase in spinach chloroplast. Photosynthesis Research **34,** 155-155.

Mizukami, Y., Huang, H., Tudor, M., Hu, Y., and Ma, H. (1996). Functional domains of the floral regulator AGAMOUS: characterization of the DNA binding domain and analysis of dominant negative mutations. The Plant Cell **8,** 831-845.

Moffat, C.S.U.o.D. (2007). Identifying signal transduction components acting downstream of reactive oxygen species (ROS) in *Arabidopsis thaliana* (Durham University).

Moller, I.M., Jensen, P.E., and Hansson, A. (2007). Oxidative modifications to cellular components in plants. Annual Review of Plant Biology **58,** 459-481.

Montillet, J.L., Chamnongpol, S., Rusterucci, C., Dat, J., van de Cotte, B., Agnel, J.P., Battesti, C., Inze, D., Van Breusegem, F., and Triantaphylides, C. (2005). Fatty acid hydroperoxides and H_2O_2 in the execution of hypersensitive cell death in tobacco leaves. Plant Physiology **138,** 1516-1526.

Muller-Moule, P., Golan, T., and Niyogi, K.K. (2004). Ascorbate-deficient mutants of arabidopsis grow in high light despite chronic photooxidative stress. Plant Physiology **134,** 1163-1172.

Murgia, I., Tarantino, D., Vannini, C., Bracale, M., Carravieri, S., and Soave, C. (2004). *Arabidopsis thaliana* plants overexpressing thylakoidal ascorbate peroxidase show increased resistance to Paraquat-induced photooxidative stress and to nitric oxide-induced cell death. The Plant Journal **38,** 940-953.

Nakano, Y., and Asada, K. (1987). Purification of ascorbate peroxidase in spinach-chloroplasts-its inactivation in ascorbate-depleted medium and reactivation by monodehydroascorbate radical. Plant & Cell Physiology **28,** 131-140.

Niki, T., Mitsuhara, I., Seo, S., Ohtsubo, N., and Ohashi, Y. (1998). Antagonistic effect of salicylic acid and jasmonic acid on the expression of pathogenesis-related (PR) protein genes in wounded mature tobacco leaves. Plant & Cell Physiology **39,** 500-507.

Niu, Y., Wang, Y.P., Li, P., Zhang, F., Liu, H., and Zheng, G.C. (2013). Drought stress induces oxidative stress and the antioxidant defense system in ascorbate-deficient *vtc1* mutants of *Arabidopsis thaliana*. Acta Physiologiae Plantarum **35,** 1189-1200.

Noctor, G., Gomez, L., Vanacker, H., and Foyer, C.H. (2002). Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signalling. Journal of Experimental Botany **53,** 1283-1304.

O'Connor, T.R., Dyreson, C., and Wyrick, J.J. (2005). Athena: a resource for rapid visualization and systematic analysis of Arabidopsis promoter sequences. Bioinformatics **21,** 4411-4413.

Oelze, M., Vogel, M., Alsharafa, K., Kahmann, U., Viehhauser, A., Maurino, V., and Dietz, K. (2012). Efficient acclimation of the chloroplast antioxidant defence of *Arabidopsis thaliana* leaves in response to a 10- or 100-fold light increment and the possible involvement of retrograde signals. Journal of Experimental Botany **63,** 1297-1313.

Ogawa, K.i., Kanematsu, S., Takabe, K., and Asada, K. (1995). Attachment of CuZn-Superoxide Dismutase to thylakoid membranes at the site of superoxide generation (PSI) in spinach chloroplasts: detection by immuno-gold labeling after rapid freezing and substitution method. Plant & Cell Physiology **36,** 565-573.

Oh, E., Zhu, J.-Y., and Wang, Z.-Y. (2012). Interaction between BZR1 and PIF4 integrates brassinosteroid and environmental responses. Nature Cell Biology **14,** 802-809.

op den Camp, R.G.L., Przybyla, D., Ochsenbein, C., Laloi, C., Kim, C., Danon, A., Wagner, D., Hideg, É., Göbel, C., Feussner, I., Nater, M., and Apel, K. (2003). Rapid Induction of Distinct Stress Responses after the Release of Singlet Oxygen in Arabidopsis. The Plant Cell **15,** 2320-2332.

Orozco-Cardenas, M.L., Narvaez-Vasquez, J., and Ryan, C.A. (2001). Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. The Plant Cell **13,** 179-191.

Orr, C.W.M. (1967a). Studies on ascorbic acid .I. factors influencing ascorbate-mediated inhibition of catalase. Biochemistry **6,** 2995-3000.

Orr, C.W.M. (1967b). Studies on Ascorbic Acid .2. Physical Changes in Catalase Following Incubation with Ascorbate or Ascorbate and Copper (2). Biochemistry **6,** 3000-3006.

Ouchane, S., Picaud, M., Vernotte, C., and Astier, C. (1997). Photooxidative stress stimulates illegitimate recombination and mutability in carotenoid-less mutants of Rubrivivax gelatinosus. EMBO Journal **16,** 4777-4787.

Panchuk, II, Zentgraf, U., and Volkov, R.A. (2005). Expression of the Apx gene family during leaf senescence of *Arabidopsis thaliana*. Planta **222,** 926-932.

Pang, C.H., Li, K., and Wang, B. (2011). Overexpression of SsCHLAPXs confers protection against oxidative stress induced by high light in transgenic *Arabidopsis thaliana*. Physiologia Plantarum **143,** 355-366.

Park, K.-Y., Jung, J.-Y., Park, J., Hwang, J.-U., Kim, Y.-W., Hwang, I., and Lee, Y. (2003). A role for phosphatidylinositol 3-phosphate in abscisic acid-induced reactive oxygen species generation in guard cells. Plant Physiology **132,** 92-98.

Payton, P., Webb, R., Kornyeyev, D., Allen, R., and Holaday, A.S. (2001). Protecting cotton photosynthesis during moderate chilling at high light intensity by increasing chloroplastic antioxidant enzyme activity. Journal of Experimental Botany **52,** 2345-2354.

Pei, Z.-M., Murata, Y., Benning, G., Thomine, S., Klusener, B., Allen, G.J., Grill, E., and Schroeder, J.I. (2000a). Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. Nature **406,** 731-734.

Pei, Z.M., Murata, Y., Benning, G., Thomine, S., Klusener, B., Allen, G.J., Grill, E., and Schroeder, J.I. (2000b). Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. Nature **406,** 731-734.

Pesaresi, P., Schneider, A., Kleine, T., and Leister, D. (2007). Interorganellar communication. Current Opinion in Plant Biology **10,** 600-606.

Pesaresi, P., Masiero, S., Eubel, H., Braun, H.-P., Bhushan, S., Glaser, E., Salamini, F., and Leister, D. (2006). Nuclear photosynthetic gene expression is synergistically

modulated by rates of protein synthesis in chloroplasts and mitochondria. The Plant Cell **18,** 970-991.

Pesaresi, P., Hertle, A., Pribil, M., Kleine, T., Wagner, R., Strissel, H., Ihnatowicz, A., Bonardi, V., Scharfenberg, M., Schneider, A., Pfannschmidt, T., and Leister, D. (2009). Arabidopsis STN7 kinase provides a link between short- and long-term photosynthetic acclimation. The Plant Cell **21,** 2402-2423.

Pfalz, J., Liebers, M., Hirth, M., Grubler, B., Holtzegel, U., Schroter, Y., Dietzel, L., and Pfannschmidt, T. (2012). Environmental control of plant nuclear gene expression by chloroplast redox signals. Frontiers in plant science **3,** 257.

Pfannschmidt, T., Nilsson, A., and Allen, J.F. (1999). Photosynthetic control of chloroplast gene expression. Nature **397,** 625-628.

Pfannschmidt, T., Allen, J., and Oelmuller, R. (2001). Principles of redox control in photosynthesis gene expression. Physiologia Plantarum **112,** 1-9.

Philippar, K., Geis, T., Ilkavets, I., Oster, U., Schwenkert, S., Meurer, J., and Soll, J. (2007). Chloroplast biogenesis: The use of mutants to study the etioplast-chloroplast transition. Proceedings of the National Academy of Sciences of the United States of America **104,** 678-683.

Pitsch, N., Witsch, B., and Baier, M. (2010). Comparison of the chloroplast peroxidase system in the chlorophyte *Chlamydomonas reinhardtii*, the bryophyte *Physcomitrella patens*, the lycophyte *Selaginella moellendorffii* and the seed plant *Arabidopsis thaliana*. BMC Plant Biology **10,** 133.

Pitzschke, A., Djamei, A., Bitton, F., and Hirt, H. (2009). A major role of the MEKK1-MKK1/2-MPK4 pathway in ROS signalling. Molecular Plant **2,** 120-137.

Pogson, B.J., Woo, N.S., Forster, B., and Small, I.D. (2008). Plastid signalling to the nucleus and beyond. Trends in plant science **13,** 602-609.

Polle, A. (2001). Dissecting the superoxide dismutase-ascorbate-glutathione-pathway in chloroplasts by metabolic modeling. Computer simulations as a step towards flux analysis. Plant Physiology **126,** 445-462.

Preston, J.C., and Hileman, L.C. (2013). Functional evolution in the plant SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SPL) gene family. Frontiers in Plant Science **4,** 80.

Preuss, M.L., Cameron, J.C., Berg, R.H., and Jez, J.M. (2014). Immunolocalization of glutathione biosynthesis enzymes in *Arabidopsis thaliana*. Plant Physiology Biochem **75,** 9-13.

Queval, G., Thominet, D., Vanacker, H., Miginiac-Maslow, M., Gakiere, B., and Noctor, G. (2009). H_2O_2-activated up-regulation of glutathione in arabidopsis involves induction of genes encoding enzymes involved in cysteine synthesis in the chloroplast. Molecular Plant **2,** 344-356.

Ramel, F., Birtic, S., Cuine, S., Triantaphylides, C., Ravanat, J.L., and Havaux, M. (2012a). Chemical quenching of singlet oxygen by carotenoids in plants. Plant Physiololgy **158,** 1267-1278.

Ramel, F., Birtic, S., Ginies, C., Soubigou-Taconnat, L., Triantaphylides, C., and Havaux, M. (2012b). Carotenoid oxidation products are stress signals that mediate gene responses to singlet oxygen in plants. Proceedings of the National Academy of Sciences of the United States of America **109,** 5535-5540.

Rapp, J.C., and Mullet, J.E. (1991). Chloroplast transcription is required to express the nuclear genes rbcS and cab. Plastid DNA copy number is regulated independently. Plant Molecular Biology **17,** 813-823.

Rauser, W.E. (1990). Phytochelatins. Annual Review of Biochemistry **59,** 61-86.

Reth, M. (2002). Hydrogen peroxide as second messenger in lymphocyte activation. Nature Immunology **3,** 1129-1134.

Rizhsky, L., Liang, H., and Mittler, R. (2003). The water-water cycle is essential for chloroplast protection in the absence of stress. The Journal of Biological Chemistry **278,** 38921-38925.

Ruckle, M.E., DeMarco, S.M., and Larkin, R.M. (2007). Plastid signals remodel light signaling networks and are essential for efficient chloroplast biogenesis in Arabidopsis. The Plant cell **19,** 3944-3960.

Rush, J.D., and Bielski, B.H.J. (1985). Pulse radiolytic studies of the reaction of perhydroxyl/superoxide O_2^- with iron(II)/iron(III) ions. The reactivity of HO_2/O_2^- with ferric ions and its implication on the occurrence of the Haber-Weiss reaction. The Journal of Physical Chemistry **89,** 5062-5066.

Rushton, P.J., Reinstadler, A., Lipka, V., Lippok, B., and Somssich, I.E. (2002). Synthetic plant promoters containing defined regulatory elements provide novel insights into pathogen- and wound-induced signaling. The Plant Cell **14,** 749-762.

Sablowski, R.W., Moyano, E., Culianez-Macia, F.A., Schuch, W., Martin, C., and Bevan, M. (1994). A flower-specific Myb protein activates transcription of phenylpropanoid biosynthetic genes. EMBO Journal. **13,** 128-137.

Sagi, M., Davydov, O., Orazova, S., Yesbergenova, Z., Ophir, R., Stratmann, J.W., and Fluhr, R. (2004). Plant respiratory burst oxidase homologs impinge on wound responsiveness and development in *Lycopersicon esculentum*. The Plant cell **16,** 616-628.

Sakai, H., Aoyama, T., and Oka, A. (2000). Arabidopsis ARR1 and ARR2 response regulators operate as transcriptional activators. The Plant Journal **24,** 703-711.

Sakamoto, W., Miura, E., and Kato, Y. (2013). A novel link between chloroplast development and stress response lessoned by leaf-variegated mutant. in photosynthesis research for food, fuel and the future (Springer Berlin Heidelberg), pp. 669-673.

Sakihama, Y., Cohen, M.F., Grace, S.C., and Yamasaki, H. (2002). Plant phenolic antioxidant and prooxidant activities: phenolics-induced oxidative damage mediated by metals in plants. Toxicology **177,** 67-80.

Salinas, M., Xing, S., Hohmann, S., Berndtgen, R., and Huijser, P. (2012). Genomic organization, phylogenetic comparison and differential expression of the SBP-box family of transcription factors in tomato. Planta **235,** 1171-1184.

Salk Insititue Genomic Analysis Laboratory. (2003). T-DNA Primer Design.

Sasaki-Sekimoto, Y., Taki, N., Obayashi, T., Aono, M., Matsumoto, F., Sakurai, N., Suzuki, H., Hirai, M.Y., Noji, M., Saito, K., Masuda, T., Takamiya, K., Shibata, D., and Ohta, H. (2005). Coordinated activation of metabolic pathways for antioxidants and defence compounds by jasmonates and their roles in stress tolerance in Arabidopsis. The Plant Journal **44,** 653-668.

Sasaki-Sekimoto, Y., Taki, N., Obayashi, T., Aono, M., Matsumoto, F., Sakurai, N., Suzuki, H., Hirai, M., Noji, M., Saito, K., Masuda, T., Takamiya, K., Shibata, D., and Ohta, H. (2006). Coordinated activation of metabolic pathways for antioxidants

and defence compounds by jasmonates and their roles in stress tolerance in Arabidopsis. Plant & Cell Physiology **47,** S233-S233.

Satoh, R., Nakashima, K., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2002). ACTCAT, a novel *cis*-acting element for proline- and hypoosmolarity-responsive expression of the *ProDH* gene encoding proline dehydrogenase in Arabidopsis. Plant Physiology. **130,** 709-719.

Satoh, R., Fujita, Y., Nakashima, K., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2004). A novel subgroup of bZIP proteins functions as transcriptional activators in hypoosmolarity-responsive expression of the *ProDH* gene in Arabidopsis. Plant Cell Physiology. **45,** 309-317.

Scheibe, R. (1981). Thioredoxin-m in pea-chloroplasts - concentration and redox state under light and dark conditions. FEBS Letters **133,** 301-304.

Scheibe, R., Backhausen, J.E., Emmerlich, V., and Holtgrefe, S. (2005). Strategies to maintain redox homeostasis during photosynthesis under changing conditions. Journal of Experimental Botany **56,** 1481-1489.

Schindler, U., Beckmann, H., and Cashmore, A.R. (1992). TGA1 and G-box binding factors: two distinct classes of Arabidopsis leucine zipper proteins compete for the G-box-like element TGACGTGG. The Plant cell **4,** 1309-1319.

Schroeder, D.F., Gahrtz, M., Maxwell, B.B., Cook, R.K., Kan, J.M., Alonso, J.M., Ecker, J.R., and Chory, J. (2002). De-etiolated 1 and damaged DNA binding protein 1 interact to regulate Arabidopsis photomorphogenesis. Current Biology **12,** 1462-1472.

Schurmann, P., and Buchanan, B.B. (2008). The ferredoxin/thioredoxin system of oxygenic photosynthesis. Antioxidants & Redox Signaling **10,** 1235-1274.

Schwab, R., Palatnik, J.F., Riester, M., Schommer, C., Schmid, M., and Weigel, D. (2005). Specific effects of microRNAs on the plant transcriptome. Developmental Cell **8,** 517-527.

Sewelama, N., Jasperta, N., Van Der Kelenc, K., Tognettic, V.B., Schmitza, J., Frerigmannd, H., Stahle, E., Zeiere, J., Van Breusegem, F., and G. Maurinoa , V. (2014). Spatial H_2O_2 Signalling Specificity: H_2O_2 from Chloroplasts and Peroxisomes Modulates the Plant Transcriptome Differentially. Molecular Plant **7,** 1191-1210.

Shaikhali, J., Heiber, I., Seidel, T., Stroher, E., Hiltscher, H., Birkmann, S., Dietz, K.-J., and Baier, M. (2008). The redox-sensitive transcription factor Rap2.4a controls nuclear expression of 2-Cys peroxiredoxin A and other chloroplast antioxidant enzymes. BMC Plant Biology **8,** 48.

Shaikhali, J., Noren, L., Barajas-Lopez, J.D., Srivastava, V., Konig, J., Sauer, U.H., Wingsle, G., Dietz, K.J., and Strand, A. (2012). Redox-mediated Mechanisms Regulate DNA binding activity of the G-group of basic region leucine zipper (bZIP) transcription factors in Arabidopsis. Journal of Biological Chemistry **287,** 27510-27525.

Sharma, P., Jha, A.B., Dubey, R.S., and Pessarakli, M. (2012). Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. Journal of Botany **2012**.

Sharp, K.H., Mewies, M., Moody, P.C.E., and Raven, E.L. (2003). Crystal structure of the ascorbate peroxidase-ascorbate complex. Nature Structural Biology **10,** 303-307.

Shi, L.-X., and Theg, S.M. (2013). The chloroplast protein import system: From algae to trees. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research **1833,** 314-331.

Shikanai, T., Munekage, Y., and Kimura, K. (2002). Regulation of proton-to-electron stoichiometry in photosynthetic electron transport: physiological function in photoprotection. Journal of Plant Research **115,** 3-10.

Shikanai, T., Takeda, T., Yamauchi, H., Sano, S., Tomizawa, K.-I., Yokota, A., and Shigeoka, S. (1998). Inhibition of ascorbate peroxidase under oxidative stress in tobacco having bacterial catalase in chloroplasts. FEBS Letters **428,** 47-51.

Simossis, V.A., and Heringa, J. (2005). PRALINE: a multiple sequence alignment toolbox that integrates homology-extended and secondary structure information. Nucleic Acids Research **33,** W289-294.

Simpson, S.D., Nakashima, K., Narusaka, Y., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2003). Two different novel *cis*-acting elements of erd1, a clpA homologous Arabidopsis gene function in induction by dehydration stress and dark-induced senescence. The Plant Journal. **33,** 259-270.

Skirycz, A., Vandenbroucke, K., Clauw, P., Maleux, K., De Meyer, B., Dhondt, S., Pucci, A., Gonzalez, N., Hoeberichts, F., Tognetti, V.B., Galbiati, M., Tonelli, C., Van Breusegem, F., Vuylsteke, M., and Inze, D. (2011). Survival and growth of Arabidopsis plants given limited water are not equal. Nature Biotechnology **29,** 212-214.

Solymosi, K., and Schoefs, B. (2010). Etioplast and etio-chloroplast formation under natural conditions: the dark side of chlorophyll biosynthesis in angiosperms. Photosynthesis Research **105,** 143-166.

Song, Y.G., Liu, B., Wang, L.F., Li, M.H., and Liu, Y. (2006). Damage to the oxygen-evolving complex by superoxide anion, hydrogen peroxide, and hydroxyl radical in photoinhibition of photosystem II. Photosynthesis Research **90,** 67-78.

Stewart, C.N., Jr., and Via, L.E. (1993). A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. BioTechniques **14,** 748-750.

Stief, A., Altmann, S., Hoffmann, K., Pant, B.D., Scheible, W.-R., and Bäurle, I. (2014). Arabidopsis miR156 regulates tolerance to recurring environmental stress through SPL transcription factors. The Plant Cell **26,** 1792-1807.

Strand, A., Asami, T., Alonso, J., Ecker, J.R., and Chory, J. (2003). Chloroplast to nucleus communication triggered by accumulation of Mg-protoporphyrinIX. Nature **421,** 79-83.

Sukrong, S., Yun, K.Y., Stadler, P., Kumar, C., Facciuolo, T., Moffatt, B.A., and Falcone, D.L. (2012). Improved growth and stress tolerance in the Arabidopsis *oxt1* mutant triggered by altered adenine metabolism. Molecular Plant **5,** 1310-1332.

Sullivan, J.A., and Gray, J.C. (1999). Plastid translation is required for the expression of nuclear photosynthesis genes in the dark and in roots of the pea *lip1* mutant. Plant Cell **11,** 901-910.

Susek, R.E., Ausubel, F.M., and Chory, J. (1993). Signal transduction mutants of Arabidopsis uncouple nuclear *CAB* and *RBCS* gene expression from chloroplast development. Cell **74,** 787-799.

Suzuki, M., Ketterling, M.G., and McCarty, D.R. (2005). Quantitative statistical analysis of *cis*-regulatory sequences in ABA/VP1- and CBF/DREB1-regulated genes of Arabidopsis. Plant Physiology **139,** 437-447.

Tamaoki, M. (2008). The role of phytohormone signaling in ozone-induced cell death in plants. Plant Signaling & Behavior **3,** 166-174.

Tang, W., and Perry, S.E. (2003). Binding site selection for the plant MADS domain protein AGL15: an *in vitro* and *in vivo* study. Journal of Biological Chemistry **278,** 28154-28159.

Tanz, S.K., Castleden, I., Hooper, C.M., Vacher, M., Small, I., and Millar, H.A. (2013). SUBA3: a database for integrating experimentation and prediction to define the SUBcellular location of proteins in Arabidopsis. Nucleic Acids Research **41,** D1185-1191.

Teixeira, F., Menezes-Benavente, L., Margis, R., and Margis-Pinheiro, M. (2004). Analysis of the molecular evolutionary history of the ascorbate peroxidase gene family: Inferences from the rice genome. Journal of Molecular Evolution **59,** 761-770.

Thalhammer, A., Bryant, G., Sulpice, R., and Hincha, D.K. (2014). Disordered cold regulated15 proteins protect chloroplast membranes during freezing through binding and folding, but do not stabilize chloroplast enzymes *in vivo*. Plant physiology **166,** 190-201.

Thannickal, V.J., and Fanburg, B.L. (2000). Reactive oxygen species in cell signaling. American Journal of Physiology - Lung Cellular and Molecular Physiology **279,** L1005-1028.

Thomsen, B., Drumm-Herrel, H., and Mohr, H. (1992). Control of the appearance of ascorbate peroxidase (EC 1.11.1.11) in mustard seedling cotyledons by phytochrome and photooxidative treatments. Planta **186,** 600-608.

Tikkanen, M., Grieco, M., Nurmi, M., Rantala, M., Suorsa, M., and Aro, E.-M. (2012). Regulation of the photosynthetic apparatus under fluctuating growth light. Philosophical Transactions of the Royal Society B: Biological Sciences **367,** 3486-3493.

Torres, M.A., Jones, J.D.G., and Dangl, J.L. (2006). Reactive oxygen species signaling in response to pathogens. Plant Physiology **141,** 373-378.

Tran, L.S., Nakashima, K., Sakuma, Y., Simpson, S.D., Fujita, Y., Maruyama, K., Fujita, M., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2004). Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive *cis*-element in the early responsive to dehydration stress 1 promoter. The Plant Cell **16,** 2481-2498.

Triantaphylides, C., Krischke, M., Hoeberichts, F.A., Ksas, B., Gresser, G., Havaux, M., Van Breusegem, F., and Mueller, M.J. (2008). Singlet oxygen is the major reactive oxygen species involved in photooxidative damage to plants. Plant Physiology **148,** 960-968.

Tripathy, B.C., and Oelmuller, R. (2012). Reactive oxygen species generation and signaling in plants. Plant signaling & behavior **7,** 1621-1633.

Urao, T., Yamaguchi-Shinozaki, K., Urao, S., and Shinozaki, K. (1993). An Arabidopsis myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. The Plant Cell **5,** 1529-1539.

Urzica, E.I., Adler, L.N., Page, M.D., Linster, C.L., Arbing, M.A., Casero, D., Pellegrini, M., Merchant, S.S., and Clarke, S.G. (2012). Impact of Oxidative Stress on Ascorbate Biosynthesis in Chlamydomonas via Regulation of the VTC2 Gene Encoding a GDP-L-galactose Phosphorylase. Journal of Biological Chemistry **287,** 14234-14245.

Usami, T., Mochizuki, N., Kondo, M., Nishimura, M., and Nagatani, A. (2004). Cryptochromes and phytochromes synergistically regulate Arabidopsis root greening under blue light. Plant Cell Physiology **45,** 1798-1808.

Valpuesta, V., and Botella, M.A. (2004). Biosynthesis of L-ascorbic acid in plants: new pathways for an old antioxidant. Trends in Plant Science **9,** 573-577.

Vanderauwera, S., Zimmermann, P., Rombauts, S., Vandenabeele, S., Langebartels, C., Gruissem, W., Inze, D., and Van Breusegem, F. (2005). Genome-wide analysis of hydrogen peroxide-regulated gene expression in Arabidopsis reveals a high light-induced transcriptional cluster involved in anthocyanin biosynthesis. Plant Physiology **139,** 806-821.

Vatamaniuk, O.K., Mari, S., Lu, Y.P., and Rea, P.A. (1999). AtPCS1, a phytochelatin synthase from Arabidopsis: Isolation and *in vitro* reconstitution. Proceedings of the National Academy of Sciences of the United States of America **96,** 7110-7115.

Vatamaniuk, O.K., Mari, S., Lu, Y.-P., and Rea, P.A. (2000). Mechanism of heavy metal ion activation of Phytochelatin (PC) Synthase: blocked thiols are sufficient for pc synthase-catalyzed transpeptidation of glutathione and related thiol peptides. Journal of Biological Chemistry **275,** 31451-31459.

Villain, P., Mache, R., and Zhou, D.X. (1996). The mechanism of GT element-mediated cell type-specific transcriptional control. Journal of Biological Chemistry **271,** 32593-32598.

Vogel, M.O., Moore, M., Konig, K., Pecher, P., Alsharafa, K., Lee, J., and Dietz, K.J. (2014). Fast retrograde signaling in response to high light involves metabolite export, MITOGEN-ACTIVATED PROTEIN KINASE6, and AP2/ERF transcription factors in Arabidopsis. The Plant Cell **26,** 1151-1165.

Wagner, D., Przybyla, D., Camp, R.O.D., Kim, C., Landgraf, F., Lee, K.P., Wursch, M., Laloi, C., Nater, M., Hideg, E., and Apel, K. (2004). The genetic basis of singlet oxygen-induced stress responses of *Arabidopsis thaliana*. Science **306,** 1183-1185.

Walker, D.A. (1971). Chloroplasts (and Grana): Aqueous (including high carbon fixation ability). In Methods in Enzymology, P. Anthony San, ed (Academic Press), pp. 211-220.

Wang, H., and Cutler, A. (1995). Promoters from *Kin1* and *Cor6.6*, two *Arabidopsis thaliana* low-temperature-and ABA-inducible genes, direct strong β-glucuronidase expression in guard cells, pollen and young developing seeds. Plant Molecular Biology **28,** 619-634.

Wang, J.W., Czech, B., and Weigel, D. (2009). miR156-regulated SPL transcription factors define an endogenous flowering pathway in *Arabidopsis thaliana*. Cell **138,** 738-749.

Wang, P.-C., Du, Y.-Y., An, G.-Y., Zhou, Y., Miao, C., and Song, C.-P. (2006a). Analysis of Global Expression Profiles of Arabidopsis Genes Under Abscisic Acid and H_2O_2 Applications. Journal of Integrative Plant Biology **48,** 62-74.

Wang, P., Duan, W., Takabayashi, A., Endo, T., Shikanai, T., Ye, J.Y., and Mi, H. (2006b). Chloroplastic NAD(P)H dehydrogenase in tobacco leaves functions in alleviation of oxidative damage caused by temperature stress. Plant Physiology **141,** 465-474.

Wang, R., Guan, P., Chen, M., Xing, X., Zhang, Y., and Crawford, N.M. (2010). Multiple regulatory elements in the Arabidopsis NIA1 promoter act synergistically to form a nitrate enhancer. Plant physiology **154,** 423-432.

Wang, Y.Y., Hecker, A.G., and Hauser, B.A. (2014). The APX4 locus regulates seed vigor and seedling growth in *Arabidopsis thaliana*. Planta **239,** 909-919.

Weigel, D., and Glazebrook, J. (2006). Transformation of agrobacterium using the freeze-thaw method. Spring Harbor Protocols **2006**.

Weiher, H., Konig, M., and Gruss, P. (1983). Multiple point mutations affecting the simian virus 40 enhancer. Science **219,** 626-631.

Welinder, K.G. (1992). Superfamily of plant, fungal and bacterial peroxidases. Current Opinion in Structural Biology **2,** 388-393.

Willingham, N., Lloyd, J., and Raines, C. (1994). Molecular cloning of the *Arabidopsis thaliana* sedoheptulose-1,7-biphosphatase gene and expression studies in wheat and Arabidopsis thaliana. Plant Molecular Biology **26,** 1191-1200.

Wise, R. (1995). Chilling-enhanced photooxidation: The production, action and study of reactive oxygen species produced during chilling in the light. Photosynthesis Research **45,** 79-97.

Wojtaszek, P. (1997). Oxidative burst: an early plant response to pathogen infection. Biochemical Journal **322 (Pt 3),** 681-692.

Woo, H.R., Kim, J.H., Kim, J., Kim, J., Lee, U., Song, I.J., Kim, J.H., Lee, H.Y., Nam, H.G., and Lim, P.O. (2010). The RAV1 transcription factor positively regulates leaf senescence in Arabidopsis. Journal of Experimental Botany **61,** 3947-3957.

Wu, C., Washida, H., Onodera, Y., Harada, K., and Takaiwa, F. (2000). Quantitative nature of the Prolamin-box, ACGT and AACA motifs in a rice glutelin gene promoter: minimal *cis*-element requirements for endosperm-specific gene expression. The Plant Journal. **23,** 415-421.

Xiao, Y., Savchenko, T., Baidoo, E.E., Chehab, W.E., Hayden, D.M., Tolstikov, V., Corwin, J.A., Kliebenstein, D.J., Keasling, J.D., and Dehesh, K. (2012). Retrograde signaling by the plastidial metabolite MEcPP regulates expression of nuclear stress-response genes. Cell **149,** 1525-1535.

Xing, M., and Xue, H. (2012). A proteomics study of auxin effects in *Arabidopsis thaliana*. Acta Biochimica et Biophysica Sinica **44,** 783-796.

Yabuta, Y., Motoki, T., Yoshimura, K., Takeda, T., Ishikawa, T., and Shigeoka, S. (2002). Thylakoid membrane-bound ascorbate peroxidase is a limiting factor of antioxidative systems under photo-oxidative stress. The Plant Journal **32,** 915-925.

Yabuta, Y., Mieda, T., Rapolu, M., Nakamura, A., Motoki, T., Maruta, T., Yoshimura, K., Ishikawa, T., and Shigeoka, S. (2007). Light regulation of ascorbate biosynthesis is dependent on the photosynthetic electron transport chain but independent of sugars in Arabidopsis. Journal of Experimental Botany **58,** 2661-2671.

Yamaguchi, K., Mori, H., and Nishimura, M. (1995). A novel isoenzyme of ascorbate peroxidase localized on glyoxysomal and leaf peroxisomal membranes in pumpkin. Plant & Cell Physiology **36,** 1157-1162.

Yoshimura, K., Yabuta, Y., Ishikawa, T., and Shigeoka, S. (2002). Identification of a *cis* element for tissue-specific alternative splicing of chloroplast ascorbate peroxidase pre-mRNA in higher plants. The Journal of Biological Chemistry **277,** 40623-40632.

Zamocky, M. (2004). Phylogenetic relationships in class I of the superfamily of bacterial, fungal, and plant peroxidases. European Journal Biochemistry **271,** 3297-3309.

Zhang, H., He, H., Wang, X., Wang, X., Yang, X., Li, L., and Deng, X.W. (2011). Genome-wide mapping of the HY5-mediated genenetworks in Arabidopsis that involve both transcriptional and post-transcriptional regulation. The Plant Journal **65,** 346-358.

Zhang, S., and Scheller, H.V. (2004). Photoinhibition of photosystem I at chilling temperature and subsequent recovery in *Arabidopsis thaliana*. Plant Cell Physiology **45,** 1595-1602.

Zhang, Z., Zhang, Q., Wu, J., Zheng, X., Zheng, S., Sun, X., Qiu, Q., and Lu, T. (2013). gene knockout study reveals that cytosolic ascorbate peroxidase 2(osapx2) plays a critical role in growth and reproduction in rice under drought, salt and cold stresses. PLoS ONE **8,** e57472.

Zimmermann, P., Hennig, L., and Gruissem, W. (2005). Gene-expression analysis and network discovery using Genevestigator. Trends in Plant Science **10,** 407-409.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L., and Gruissem, W. (2004). GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiology **136,** 2621-2632.

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POSTER ABSTRACT

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I hereby declare that I have prepared and written the presented doctoral thesis myself using only the presented methods and sources. All sources from literature are marked as such and are properly cited

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