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FACULTY OF SCIENCES – DEPARTMENT MOLECULAR GENETICS
VIB DEPARTMENT OF PLANT SYSTEMS BIOLOGY

ROLE FOR HYDROGEN PEROXIDE DURING ABIOTIC AND BIOTIC STRESS SIGNALING IN PLANTS

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The scientist is not a person who gives the right answers, he's one who asks the right questions

Claude Lévi-Strauss

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LIST OF ABBREVIATIONS

	26S RSU	26S proteasome regulatory subunit
A	ABA	Abscisic acid
	AFLP	Amplified fragment length polymorphism
	AGI	*Arabidopsis* Genome Initiative
	Agrobacterium	*Agrobacterium tumefaciens*
	APX	Ascorbate peroxidase
	Arabidopsis	*Arabidopsis thaliana*
	ATP	Adenine triphosphate
B	BLAST	Basic local alignment and search tool
C	CAT	Catalase
	cDNA	Complementary DNA
	Col	Columbia ecotype of *Arabidopsis thaliana*
D	DNA	Deoxyribonucleic acid
	DRP	DNA-afhankelijk RNA polymerase
	DW	Dry Weight
E	EST	Expressed sequence tag
F	FC	Fold Change
	FW	Fresh Weight
G	GO	Gene ontology
	GPX	Glutathione peroxidise
	GST	Gene-specific tag
	GTP	Guanine triphosphate
H	H_2O_2	Hydrogen peroxide
	HRM	H_2O_2-response matrix
	HSP	Heat shock protein
M	MAPK	Mitogen-activated protein kinase
	MI	*Myo*-inositol
	mRNA	Messenger RNA
O	OE	Overexpression
P	PCR	Polymerase chain reaction
	PDS	Phyotene desaturase
	Ph.D.	*Phylosophiae doctor*
	POF	Protein with obscure features
R	RNA	Ribonucleic acid
	ROS	Reactive oxygen species
S	SDR	Short-chain dehydrogenase/reductase
	SOD	Superoxide dismutase
	STG	Stress tolerance gene
T	T-DNA	Transferred DNA
	TF	Transcription factor
	ThrRS	Threonyl tRNA synthetase
	tRNA	Transfer RNA
	TRV	Tobacco rattle virus
U	Ub	Ubiquitin
V	VIGS	Virus-induced gene silencing
W	WIWAM	Weighing imaging and watering machine
	WT	Wild type

RESEARCH OBJECTIVES

Plants are continuously exposed to a variety of environmental conditions, abiotic (high salinity, drought, heavy metals pollution, extreme temperatures…) or biotic (pathogens), that limit their growth and productivity. Such conditions are commonly referred to as environmental stress.

A central event during (nearly) all environmental stresses is the accumulation of reactive oxygen species (ROS). ROS, including hydrogen peroxide (H_2O_2), are toxic at high concentrations, but at lower concentrations, they act as signal molecules that control the expression of genes involved in diverse developmental programs, including defense responses to stress. At the beginning of my Ph.D., a significant amount of H_2O_2-related expression data was available and it was assumed that H_2O_2-induced genes are involved in H_2O_2 signal transduction and/or defense response of plants. However, before going into a detailed study, it is necessary to reduce, in a well considered manner, the large number of H_2O_2-induced genes to a workable selection of interesting candidates genes. Therefore, the main objective of the first part of the thesis (**Chapter 2-4**) was to take advantage of existing H_2O_2-related expression data and hunt for genes that would be relevant candidates to study H_2O_2 signal transduction and plant defense responses in more detail. To do this, different strategies were pursued.

In a first, *in silico* approach (in collaboration with Prof. van de Peer), we hypothesized that genes with a conserved H_2O_2-induction could be master regulators of H_2O_2-signal transduction and we therefore wanted to assess the evolutionarily conservation of the H_2O_2-induced transcriptional response of distant species (**Chapter 2**). To search for H_2O_2-induced genes that can be candidates for the improvement of stress resistance of plants, we performed two functional screens, using putative important H_2O_2-induced genes from two different plant species: *Nicotiana tabacum* (tobacco) and *Arabidopsis thaliana*. We focused on genes encoding transcription factors and other proteins with putative regulatory functions, since such proteins are potential central regulators of plant defense responses. A first functional screen was performed in collaboration with the laboratory of phytopathology (Prof. Höfte, Department of Crop Protection), of which the main interest is the defense response of plants to necrotrophic pathogens. Since it is known that necrotrophic pathogens modulate the H_2O_2-dependent defense response of plants to kill plant cells, we evaluated genes that were involved in H_2O_2-induced cell death in tobacco for a possible role in the defense response against two of the most important necrotrophic pathogens, *Botrytis cinerea* and *Sclerotinia sclerotiorum* (**Chapter 3**). In a second screen, various transgenic *Arabidopsis* plants with perturbed levels of H_2O_2-induced genes with a possible role in the ROS signaling network of plants were assayed for altered tolerance to oxidative stress (**Chapter 4**). The goal of

these functional screens was to select interesting candidate genes and to further study their function during H_2O_2 signal transduction and environmental stress responses.

To get a better view on the complex defense response of plants to environmental stresses, we performed a detailed literature study on genes that function during stress tolerance (**Chapter 1**). We limited this study to genes involved in abiotic stress since it was the major topic of the second part of the thesis (**Chapter 5-6**). We were particularly interested in the molecular mechanisms that regulate growth during drought stress, as it is one of the greatest global constraints for agriculture. To monitor plant growth under limited watering conditions, a semi-automated platform was developed (**Chapter 5**)**.** This system will be used to evaluate the growth performance of stress-tolerant plants during drought stress. Finally, to study more in detail the molecular mechanism underpinning plant growth during drought stress, microarray analysis of drought tolerant transgenic plants were performed (**Chapter 6**).

PART I

GENERAL INTRODUCTION

CHAPTER 1

How do plants deal with abiotic stress?

Meta-analysis on transgenic plants with increased stress tolerance

ABSTRACT

Abiotic stresses negatively affect plant yield thereby causing enormous losses in agriculture worldwide, a problem which has increased the need for better adapted varieties. Major advances in understanding plant stress responses have been achieved using *Arabidopsis thaliana* as a model system. *Arabidopsis* has been successfully exploited as host species to evaluate the effect on stress tolerance caused by altered expression levels of a gene of interest. Genome-wide microarray analysis on *Arabidopsis* indicated that the plant's stress responses are tightly controlled by complex transcriptional networks controlled by stress-inducible transcription factors (TFs), which regulate the expression of genes encoding proteins that are involved in stress tolerance. A major challenge will be to integrate all data on stress tolerant plants in order to understand the stress response of plants at the systems biology level, and to overcome the difficulties that are associated with genetic engineering and limit economically successful applications for stress-tolerant crops.

INTRODUCTION

A growing world population with increased social standards combined with the urgent need for a more sustainable agriculture does not only plead for the development of crop varieties with increasing yield potential, but also for varieties that are able to cope with fluctuating and adverse environmental conditions that limit plant growth and productivity, which are referred to as abiotic stresses. These include drought, high or low temperatures, and salinity.

When a plant is exposed to abiotic stress, the expression of many genes is altered to induce protection against the negative effects of the stress. It has now become clear that increased protection involves a complex regulatory network that mediates morphological, physiological, biochemical and molecular changes. Understanding such changes has been of key importance for breeding plant resistance to abiotic stress. Breeding crop varieties with improved performance under suboptimal growing conditions is now one of the ambitious, but crucial objectives in modern plant biotechnology. Plant biotechnologists have been reporting genetically modified plant with increased stress tolerance for almost two decades.

Here, we will discuss genes that positively affect stress tolerance of plants, called stress tolerance genes (STGs), as a result of alterations in their expression levels. Due to improved plant transformation techniques, high throughput screenings, and the invention of microarrays, we have witnessed a spectacular upsurge in the number of STGs. By the end of 2007, approximately 350 different STGs had been reported, mostly conferring tolerance to salt, drought and cold/freezing stresses (Figure 1). The dramatic increase in reported STGs reflects both the augmented economical potential of stress tolerant plant varieties and the vastly improved knowledge on the underlying mechanisms controlling plant responses to abiotic stress. In the next sections, we will present a comprehensive discussion on reported STGs, with a particular emphasis on: (i) *Arabidopsis thaliana* as a model system, (ii) different approaches for engineering stress tolerance, with a focus on the recent progress made using TFs, (iii) microarray analysis for the identification and validation of STGs.

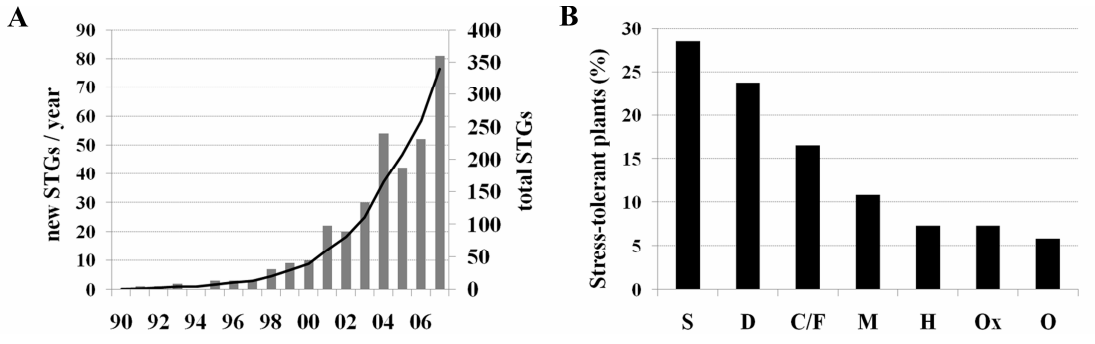

Figure 1

Reported stress tolerance genes (STGs). **A** Numbers of reported STGs over the last 17 years. Grey bars represent the numbers of new STGs each year, the black line indicates the total number of STGs. **B** Frequency distribution of reported stress tolerance for the transgenic lines. Genes that induce cross-tolerance were assigned to each of the stresses to which tolerance was described. C/F, cold/freezing; D, drought; H, heat; M, metal; O, osmotic; Ox, oxidative; S, salt.

Arabidopsis as a model system

The requirements for a good model species for genetic research at a molecular and systems level include the availability of the complete genomic sequence, easy transformation protocols, short generation times, manageable size, sizeable research community, microarray and proteomics data, and the availability of a large set of mutants. Initially, *Nicotiana tabacum* (tobacco) was used as model system for plant research and until now, it represents approximately 20% of transgenic lines with an increased stress tolerance phenotype. However, tobacco does not fulfill the requirements for a suitable model system. All requirements for a model species are present for *Arabidopsis* and the vast amount of molecular data have made *Arabidopsis* the system of choice for molecular and system-wide plant studies of abiotic stress (Salt, 2004). Approximately 45% of all currently reported STGs were *Arabidopsis* genes, while nearly 50% of all STGs have been characterized using *Arabidopsis* as the transgenic species (Figure 2).

To date, there is still no satisfactory experimental crop model system available, but for economical reasons, several crop species are now the subject of large research efforts. Sequencing of the rice genome has been recently completed, the maize genome has been presented and that of tomato is on the way. The monocot rice is related to other important crop species such as wheat and barley, and might be a more relevant model system than the dicot *Arabidopsis*. Next to improving food crops, it will also become increasingly important to apply results from simple model plant species to dedicated bio-energy plants. These include several grasses, poplar, corn and sugarcane. *Brachypodium distachyon* is a new emerging model for grasses and several major research centers (e.g. John Innes Center, l' Institute National de la Recherche Agronomique) have recently initiated research programs on it. *Brachypodium* has potential to serve as a model plant: it is small and has a small genome, transformation with biolistics or *Agrobacterium* is possible, and an EST sequencing and functional genomics project is initiated (Vogel *et al*., 2008). The genome has been sequenced by the US Department Of Energy (Joint Genome Institute) and a complete annotation is on the way. Given that *Brachypodium* is closely related to other grasses, results obtained in it can be extrapolated to almost all of the economical important grass species.

Despite the increasing interest in food, feed and bio-energy crops, *Arabidopsis* is still and will (considering the vast amount of publicly available resources) continue to be the model plant of choice. Particularly the possibilities of network elucidation via system biology approaches in

Arabidopsis are unprecedented. The next part of this introduction will focus on transgenic *Arabidopsis* plants with increased stress tolerance due to altered levels of endogenous STGs.

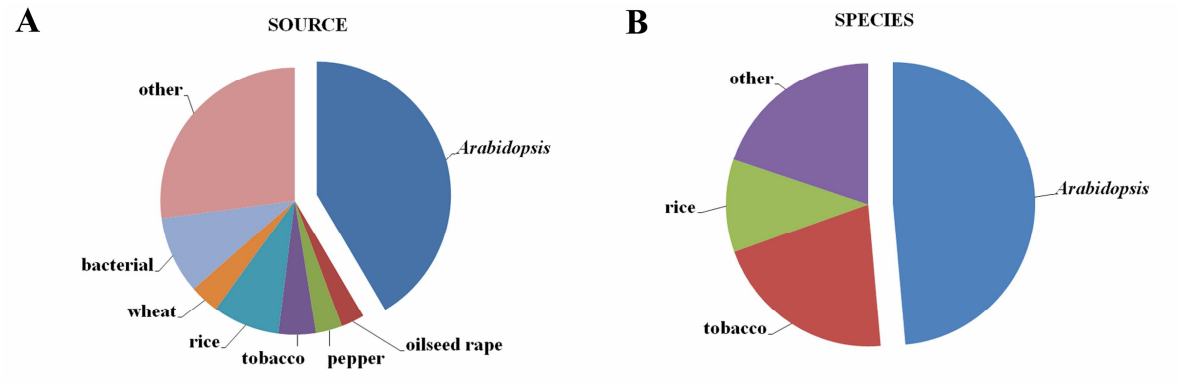

Figure 2

Classification of reported stress tolerance genes (STGs). **A** Pie diagram illustrating distribution of the different plant sources from which the STGs were isolated. **B** Pie diagram showing the distribution of the transgenic species in which the expression of the STG was modified to study stress tolerance. Other include genes sources or host species that are represented less than 2 and 10 %, respectively.

Arabidopsis STGs control various mechanisms

Excellent reviews on the molecular basis of stress tolerance in plants were published (Hasegawa *et al.,* 2000; Iba, 2002; Ingram and Bartels, 1996; Seki *et al*., 2007; Shinozaki and Yamaguchi-Shinozaki, 2007; Thomashow, 1999; Umezawa *et al*., 2006; Yamaguchi-Shinozaki and Shinozaki, 2006; Zhu, 2002). The current understanding is that stress tolerance is controlled by an extensive transcriptional regulatory network. Therefore, STGs could be identified through their stress-inducible expression. Such stress-inducible genes can be broadly classified in two groups: the first group encodes proteins that function in stress tolerance, such as molecular protectants, detoxifying proteins and ion transporters, while the second group is comprised of regulatory proteins, including enzymes involved in (phospho)lipid metabolism, protein kinases, protein phosphatases, calcium/calmodulin-binding proteins and various TFs (Figure 3*A*; Shinozaki and Yamaguchi-Shinozaki, 2007).

Until now, at least 150 *Arabidopsis* STGs have been identified that increase tolerance to abiotic stresses when their expression was altered (Supplementary Table S1). All *Arabidopsis* STGs were manually categorized by using controlled vocabularies, based on Gene Ontology (http://www.geneontology.org), and simplified vocabularies, such as the Plant GO slim

(http://www.geneontology.org/GO.slims.shtml), which allowed us to identify broad functional categories in terms of either molecular function or biological process (Figure 3*B*).

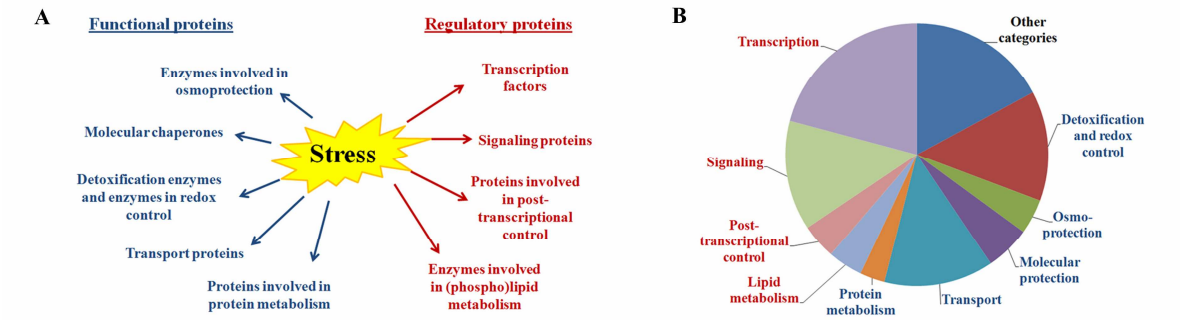

Figure 3

Functional classification of stress tolerance genes (STGs). **A** STGs are classified into two groups. The first group includes proteins that can function in stress tolerance (functional proteins), while the second group contains proteins that are involved in signal perception, transduction and gene expression and function in regulation of the stress response (regulatory proteins) **B** Pie diagram showing the functional distribution of all *Arabidopsis* STGs. Other categories include molecular functions that are represented less than 1 %.

Detoxifying genes

Abiotic stresses induce the accumulation of reactive oxygen species (ROS) which results in oxidative stress (Apel and Hirt, 2004; Laloi *et al*., 2004). ROS are extremely reactive, allowing them to undergo uncontrollable and damaging reactions with cellular components, including DNA, lipids and proteins, which can aggravate the detrimental effects of the initial stress and even lead to cell death (Halliwell, 2006; Van Breusegem and Dat, 2006). To protect against oxidative stress, plant cells possess an extensive ROS scavenging network, which involves non-enzymatic antioxidants, including vitamin C, vitamin E, glutathione, carotenoids and flavonoids, as well as numerous enzymatic mechanisms such as multiple superoxide dismutases (SOD), catalases, ascorbate peroxidases (APX), glutathione peroxidases (GPX), alternative oxidases, and peroxiredoxines (Halliwell, 2006). It was hypothesized that alleviation of oxidative damage by the use of ROS scavengers would enhance plant resistance and this was confirmed by a number of transgenic improvements using this detoxification strategy. Stress tolerance could be conferred by either direct scavenging of ROS or by enhanced removal of oxidative damaged and hazardous components of the cell. Since the accumulation of ROS and derivatives thereof is a common theme during most, if not all, abiotic stresses, the detoxification strategy enabled the generation of transgenic lines with tolerance to multiple stresses at the same time (Badawi *et al*., 2004; Basu *et al*., 2001; Gaber *et al*.,

2006; Gong *et al*., 2005a; Yoshimura *et al*., 2004; Murgia *et al*., 2004; Oberschall *et al*., 2000; Sunkar *et al*., 2006; Yamamoto *et al*., 2005; Zhao and Zhang, 2006).

In *Arabidopsis*, enhanced stress resistance in transgenic plants was achieved by increasing the levels of GPX, APX, different SOD isoforms, and enzymes involved in vitamin B6 and vitamin C biosynthesis (Miao *et al*., 2006; Sunkar *et al*., 2006; Wang *et al*., 2004; Titiz *et al*., 2006; Yamamoto *et al*., 2005). SOD catalyses the dismutation of superoxide into oxygen and hydrogen peroxide (H_2O_2) and transgenic plants with increased manganese SOD (AT3G56350) levels were shown to be more tolerant to salt stress (Wang *et al*., 2004). The biochemical functions of GPX are the reduction of H_2O_2 to water and the reduction of lipid hydroperoxides to their corresponding alcohols (Dixon *et al*., 1998). Ectopic expression of GPX3 (AT2G43350) increased stress tolerance to drought and osmotic stress, while its mutation resulted in the opposite effect on stress tolerance (Miao *et al*., 2006). As GPX, also APX can catalyze the reduction of H_2O_2 to water and it was shown that thylakoid-bound APX (AT1G77490) can protect the chloroplast from oxidative stress (Murgia *et al*., 2004; Tarantino *et al*., 2005).

As ROS can haphazardly assault any cellular component (leading to the accumulation of toxic derivatives, such as oxidized aminoacids and aldehydes), a second detoxification strategy tries to improve stress tolerance by the generation of transgenic plants that efficiently remove such molecules (Kotchoni *et al*., 2006; Kwon *et al*., 2007; Sunkar *et al*., 2003). Aldehydes, which accumulate due to side reactions of ROS with lipids and proteins, can be removed via oxidation to the corresponding alcohols by aldehyde dehydrogenases (Kirch *et al*., 2004), and ectopic expression of aldehyde dehydrogenases, ALDH3 (AT4G34240) and ALDH7 (AT1G54100), in *Arabidopsis* resulted in increased stress tolerance (Sunkar *et al*., 2003; Kotchoni *et al*., 2006). The transgenic plants not only showed improved tolerance to oxidative stress (H_2O_2, heavy metals), but also to dehydration (salt, drought), thereby suggesting that aldehyde dehydrogenase can help to maintain membrane integrity under osmotic stress.

Molecular chaperones: Heat shock and late embryogenesis abundant (LEA) proteins

One major detrimental effect of abiotic stresses is that these usually cause protein dysfunction through denaturation and aggregation of non-native proteins. Maintaining proteins in their functional conformations is important for cell survival under stress and this can accomplished through transcriptional induction of genes encoding heat shock proteins (HSPs, Wang *et al*., 2004). HSPs control the proper folding and conformation of both structural (e.g. cell membrane) and functional (e.g. enzymes) proteins and this important function has prompted researches to create transgenic lines with increased HSP levels. Studies on such plants have mostly focused on heat

stress (Katiyar-Agarwal *et al*., 2003; Guo *et al*., 2007; Hong and Vierling, 2000; Lee and Schöffl, 1999; Malik *et al*., 1999; Park and Hong, 2002; Queitsch *et al*., 2002; Rhoads *et al*., 2005; Yang *et al*., 2006). For example, increasing the levels of HSP101 (AT1G74310) in *Arabidopsis* resulted in increased tolerance to short exposure to 45°C (Queitsch *et al*., 2000). However, HSPs not only function in tolerance against heat stress. Sun and coworkers (2001) reported that increasing the levels of HSP17.6A (AT5G12030) successfully improved tolerances to other than heat stress, including drought and salt stress.

In addition to HSPs, also LEA-type proteins can confer molecular protection of cellular components during abiotic stress (Wang *et al*., 2003). LEA-type proteins are encoded by RD (responsive to dehydration), ERD (early responsive to dehydration), KIN (cold inducible), COR (cold regulated), and RAB (responsive to abscisic acid) genes in different plant species (Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2002). As HSPs are typically induced by high temperatures, LEA proteins accumulate in response to dehydration (drought, osmotic and/or cold stress). The actual function of these proteins remains however largely unknown. Their hydrophilicity suggest that LEA proteins act as water-binding proteins, but additional functions, including ion sequestration and protein and membrane stability, have also been proposed (Thomashow, 1998, 1999). It is known that the expression of COR genes is induced by low temperatures to confer protection against freezing stress. Ectopic expression of COR15A (AT2G42540) was shown to protect chloroplast against freezing stress by membrane stabilization (Artus *et al*., 1996; Steponkus *et al*., 1998). Recently, enhancing the expression of LEA5 (AT4G02380) in *Arabidopsis* rendered plants more tolerant to oxidative stress, but also more sensitive to drought stress (Mowla *et al*., 2006).

Osmoregulation and protection by genes involved in metabolite biosynthesis:

One of the oldest approaches for genetic engineering of stress tolerance in plants (reports dating from the early 90ies) was to increase the synthesis of metabolites that protect cellular components from the detrimental effects caused by osmotic stress (Delauney and Verma, 1993; Tarczynski *et al*., 1993). Such metabolites are called osmoprotectants and include a variety of organic compounds, such as sugars and sugar alcohols (e.g. mannitol, trehalose and galactinol), amines (e.g. polyamines and glycine betaine), and amino acids (e.g. proline) (Hasewaga *et al.,* 2000; Vinocur and Altman, 2005). These molecules do normally not interfere with cellular function and are therefore often referred to as compatible solutes.

Many plants lack the ability to synthesize the special osmoprotectants that naturally accumulate in stress tolerant species. Therefore, most transgenic approaches to increase the synthesis of osmoprotectants used bacterial biosynthetic genes, such as *Cod*A and *Bet*A (glycine

betaine), *Mtl*D (mannitol), and genes from the ectoine or trehalose biosynthesis operon. Alternatively, key biosynthetic genes, including betaine aldehyde dehydrogenase and choline monooxygenase (glycine betaine biosynthesis), and pyrroline carboxylate synthase (proline synthesis), were isolated from specific plant species, such as *Vigna aconitifolia* or *Spinacia oleracea*.

In *Arabidopsis*, knock down of an gene encoding a proline dehydrogenase (AT3G30775), which is involved in proline degradation, resulted in increased free proline accumulation and better growth performance under salt and freezing stress (Nanjo *et al*., 1999). Two other mutants, *lwr1* and *lwr2*, which are affected in proline metabolism, showed altered tolerance to osmotic stress (Verslues and Bray, 2004). Transgenic plants with increased tolerance to drought stress were also obtained by increasing the levels of raffinose family oligosaccharides through ectopic expression of an enzyme necessary for galactinol biosynthesis, GOLS2 (AT1G56600) (Taji *et al*., 2002).

The accumulation of compatible solutes during stress is important for osmoregulation and for the cellular protection by maintaining protein structures, but it may also be important for reducing or preventing the damaging effects of reactive oxygen species (ROS) (Diamant *et al*., 2001; Hare *et al*., 1998).

Ion homeostasis by transport proteins

Ion transport proteins are involved in re-establishing ionic homeostasis after salt stress by increasing ion storage in the vacuole, or by improving ion excretion from the cells (Tuteja *et al*., 2007). Different types of ion transporters, depending on their localization and selectivity, have been the target of genetic engineering. These include both vacuolar and membrane Na^+/H^+ antiporters, vacuolar $Ca^{2+/}H^+$ antiporter, and Mg^{2+}, Na^+/K^+, and Ca^{2+} transporters (Tuteja *et al*., 2007; Wang *et al*., 2003).

In *Arabidopsis*, well known stress tolerance genes encoding vacuolar ion transporters include NHX1 (AT5G27150) and AVP1 (AT1G15690). Transgenic *Arabidopsis* plants with increased levels of NHX1 exhibited substantially enhanced salt tolerance, while ectopic expression of the AVP1 H^+-transporting pyrophosphatase pump increased both salt and drought tolerance (Apse *et al*., 1999; Gaxiola *et al*., 2001).

Other ion transporters, including SOS1 (AT2G01980), exert their function at the plasma membrane. Ectopic expression of SOS1 was found to provide a greater proton motive force that is necessary for elevated Na^+/H^+ antiporter activities and tolerance to salt stress (Shi *et al*., 2003). Plasma membrane cation/proton antiporters (such as SOS1) cause alkalinization of the apoplast, thereby changing the activity and conformation of membrane proteins which might serve as a signal to mediate gene regulation and induce a general stress response (Chung *et al*., 2008).

Lipid metabolism and signaling

Adaptation of living cells to low temperatures involves alterations in the membrane lipid composition, for example by decreasing membrane fluidity through fatty acid unsaturation. Therefore, increasing the number of unsaturated fatty acids by genetic engineering could improve stress tolerance in plants (Ariizumi *et al*., 2002; Khodakovskaya *et al*., 2006; Orlova *et al*., 2003; Sui *et al*., 2007; Zhang *et al*., 2005).

In *Arabidopsis*, several attempts to increase stress tolerance by altering lipid metabolism involved phospholipase D (PLD), which hydrolyzes membranes resulting in membrane dysfunction and the production of lipid-derived signaling molecules, such as phosphatidic acid (PA). At least two different PLD isoforms, PLDα and PLDδ, with separate roles during freezing tolerance were described (Li *et al*., 2004; Welti *et al*., 2002; Rajashekar *et al*., 2006). Suppression of PLDα rendered plants more tolerant to freezing stress which was correlated with increased expression of COR genes (Welti *et al*., 2002; Rajashekar *et al*., 2006). In contrast, suppression of PLDδ results in increased sensitivity (Li *et al*., 2004). The observed discrepancies between PLDα and PLDδ can probably be explained by differences in cellular functions. In contrast to PLDδ, which is located in the plasma membrane, PLDα is located in both plasma membrane and intracellular membranes and is responsible for most of the released phosphatidic acid. It is likely that differences in levels, timing, and location of PA produced by PLD are responsible for different outcomes in stress tolerance (Li *et al*., 2004).

Regulation of gene expression by TFs and other regulatory genes

Probably the most important strategy for engineering abiotic stress tolerance in plants relies on the expression of genes that are involved in signaling and regulatory pathways (Seki *et al*., 2003, Shinozaki *et al*., 2003). The use of TFs for tailoring stress tolerance is often referred to as regulon biotechnology because it affects the expression of many genes together (Nakashima and Yamaguchi-Shinozaki, 2005; Umezawa *et al*., 2006). Figure 3 shows that approximately 20% of all STGs are involved in transcription, illustrating the importance of transcriptional reprogramming during stress-adaption. One of the reasons for their popularity is that TFs are believed to mediate durable tolerance to multiple stresses.

Most TFs that control stress tolerance in *Arabidopsis* belong to (large) protein families. These families include APETALA2/ethylene response factors (AP2/ERF) such as the DREB/CBF (drought-responsive element binding/cold-responsive element binding factor) proteins, basic-domain leucine-zipper (bZIP) proteins such as ABFs (abscisic acid (ABA)-responsive element binding factor), basic helix-loop-helix proteins (including MYC proteins), NAC (petunia NAM *Arabidopsis* ATAF1/2,

and CUC2-domain) proteins, MYB-related proteins, as well as different families of zinc-fingers domain-containing proteins, such as WRKY binding factors, C3H- and C2H2-type TFs (Figure 4). The function of several *Arabidopsis* TFs, including DREB1/CBF, DREB2, ABF2-4, RD26, MYC2, MYB2, is known since a long time and their regulatory role during stress tolerance is well-characterized. Since many excellent reviews have been published on these TFs over the last years, they will not be discussed in detail here (Agarwal, 2006; Thomashow, 1999; Nakashima and Yamaguchi-Shinozaki, 2005; Umezawa *et al*., 2006; Yamaguchi-Shinozaki and Shinozaki, 2006). Instead, special attention will be given to new discovered TFs and their roles during stress tolerance in *Arabidopsis*.

Recently identified transcriptional regulators that function during stress tolerance include MBF1A (multi-protein bridging factor 1a; AT2G42680), NF-YB1 (nuclear factor YB1; AT2G38880), HARDY (AT2G36450) and SZF1/2 (Karaba *et al*., 2007; Kim *et al*., 2007a; Nelson *et al*., 2007; Sun *et al*., 2007). NF-YB1 was one of the ~40 TFs that were identified through a large-scale functional genomics program performed on >1500 *Arabidopsis* TFs to identify regulators of drought tolerance (Nelson *et al*., 2007). It encodes a subunit of the heterotrimeric NF-Y complex that belongs the HAP/CAAT family of TFs and was found to regulate drought tolerance independent of CBF or ABA pathways (Nelson *et al*., 2007). A phenotypic screen of an activation-tagged mutant collection in *Arabidopsis* led to the discovery of HARDY, an AP2/ERF-like TF with probably unique functions during drought tolerance (Karaba *et al*., 2007). MBF1A encodes a transcriptional co-activator and its ectopic expression led to elevated salt tolerance, resistance to fungal disease and glucose insensitivity of transgenic lines (Kim *et al*., 2007a). Another MBF1 protein, MBF1C (AT3G24500), had earlier been shown to regulate tolerance to various stresses, including salt, heat, osmotic, high light and disease (Suzuki *et al*., 2005). The function of both MBF1 proteins (three isoforms exist in *Arabidopsis*) is mediated through perturbation or activation of ethylene responses (Kim *et al*., 2007a; Suzuki *et al*., 2005). Two new C3H-type zinc finger proteins, SZF1 and SZF2, act as negative regulators of salt tolerance by inhibiting the transcriptional induction of salt-responsive defense genes (Sun *et al*., 2007).

Upstream of TFs, stress signal transduction in plants is controlled by a multistep component systems consisting of several receptor protein kinases, calcium sensors and calcium (Ca^{2+})-dependent protein kinases, and mitogen-activated protein kinase (MAPK) cascades (Knight and Knight, 2001; Umezawa *et al*., 2006). These pathways are controlled by protein (de)phosphorylation, changes in Ca^{2+} fluxes, the accumulation of ROS and increased biosynthesis of stress hormones such as ABA (Figure 4).

A recent gain- and loss-of-function study of three cytokinin receptor histidine kinases, AHK1-3 (*Arabidopsis* histidine kinase 1-3), showed that AHK1 (AT2G17820) is a positive regulator of drought

tolerance, while AHK2 (AT5G35750) and AHK3 (AT1G27320) negatively regulate tolerance to drought and salt stress by interfering with stress- and ABA-induced defense responses (Tran *et al*., 2007). Also MAPK kinase kinase 9 (AT1G73500) is a negative regulator of stress tolerance and mutation resulted in increased tolerance to ABA, salt and osmotic stress (Alzwiy *et al*., 2007). Similarly, mutations in two calcium signaling protein kinases, CIPK23 (calcineurin B-like-interacting protein kinase; AT1G30270) and CPK23 (calcium-dependent protein kinase, AT4G04740) significantly increased tolerance to drought and/or salt stress, which was explained by altered K^+ uptake by the roots (Cheong *et al*., 2007; Ma *et al*., 2007). Evidence that protein kinases involved in stress tolerance not always act in signaling pathways controlling gene expression was provided by a study on TOR (target of rapamycin) kinase, which positively regulates plant growth and tolerance to osmotic stress by controlling translation of mRNA transcripts (Deprost *et al*., 2007).

Post-transcriptional control by RNA-binding proteins

Post-transcriptional control of stress gene expression is mediated by proteins that are involved in splicing, export and degradation of gene transcripts, which contributes to correct function of the encoded proteins. For example, increased salt tolerance by overexpression of SOS1 is dependent on ROS-induced stabilization of SOS1 mRNA transcripts (Chung *et al*., 2008). Also post-transcriptional control of antioxidant gene expression is very important in plants, as shown for APX during programmed cell death and drought stress, and for Cu/Zn SOD during tolerance against oxidative stress (Mittler *et al*., 1998; Mittler and Zilinskas 1994; Sunkar *et al*., 2006).

Several STGs that encode proteins involved in post-transcriptional processing have been identified. *Arabidopsis* SR-like 1 (AT5G37370) encodes a RNA splicing protein that increases salt tolerance, probably by interacting with and stabilizing proteins of the spliceosome (Forment *et al*., 2002). Mutations in two DEAD-box RNA helicases, STRS1 (AT1G31970) and STRS2 (AT5G08620), enhanced tolerance to multiple stresses, including salt, osmotic and heat stress, as a result of increased accumulation of stress-responsive transcripts encoding defense proteins (Kant *et al*., 2007). Other RNA-binding proteins, RZ-1a (AT3G26420) and LOS4 (AT3G53110), can act as both negative and positive regulators of stress tolerance, depending on the type of stress. Ectopic expression of a RZ-1A, encoding a zinc finger-containing glycine-rich RNA-binding protein, leads to increased tolerance to low temperatures, but also resulted in sensitivity towards drought and salt stress, while mutation of LOS4, encoding a DEAD-box RNA helicase, resulted in cold tolerance and heat sensitivity (Gong *et al*., 2005b; Kim *et al*., 2005; Kim *et al*., 2007b).

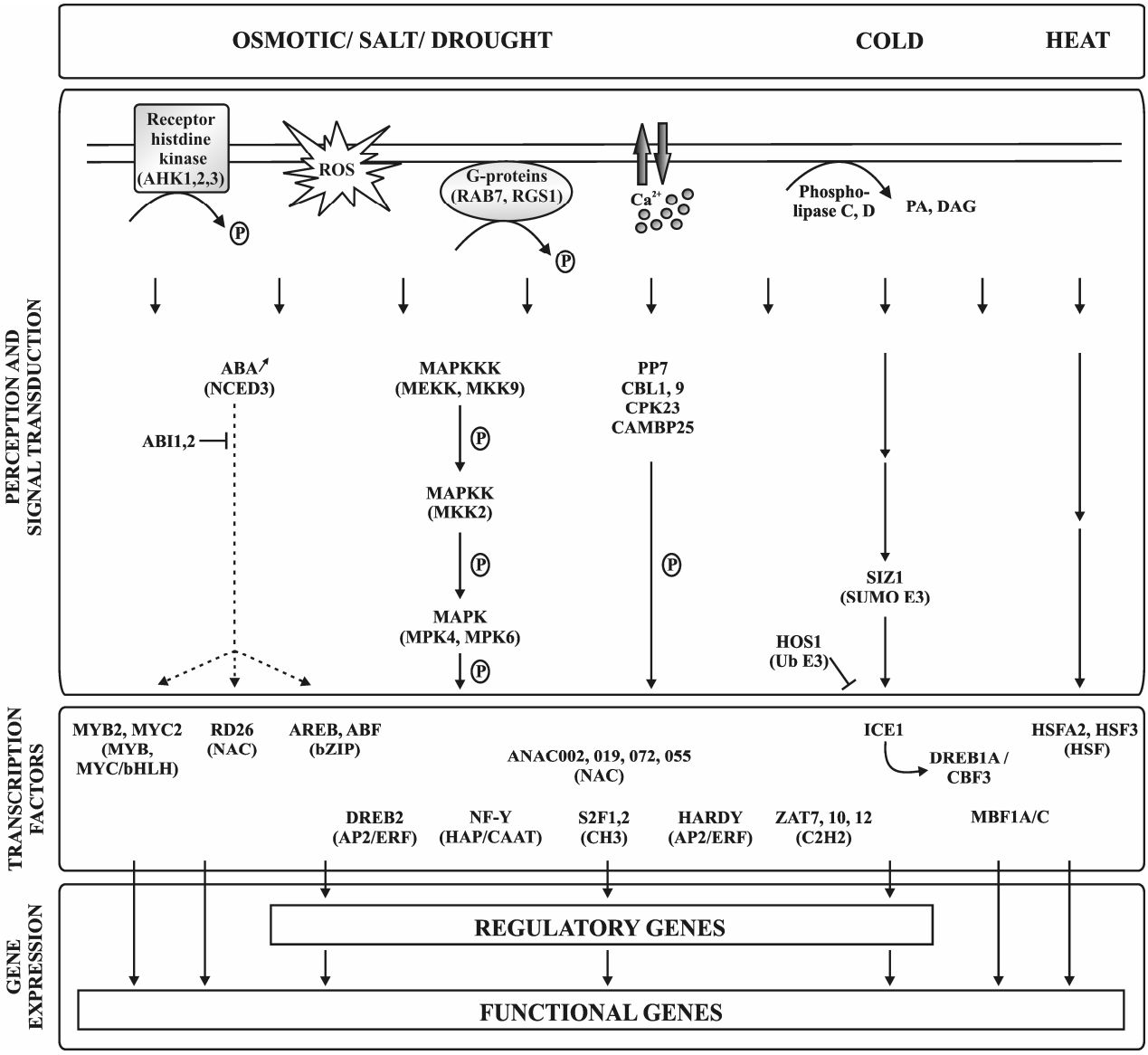

Figure 4

Transcriptional regulatory networks during abiotic stress in plants. Signal transduction pathways in drought, osmotic, salt, cold and heat stress responses consist of signal perception and signal transduction, followed by the activation of transcription factors that control the expression of defense genes involved in signal amplification (regulatory genes) and stress tolerance (functional genes). Abscisic acid (ABA)-dependent pathways are indicated by dotted lines. PA, phosphatidic acid; DAG, diacylglycerol; ROS, reactive oxygen species

Transcriptional networks during stress: Identification of STGs based on their stress-induced expression

Plant adaptation to environmental stresses involves molecular networks controlled by TFs that bind to specific regulatory elements in the promoter of defense genes (Chinussamy *et al*., 2004; Yamaguchi-Shinozaki and Shinozaki, 2005; Yamaguchi-Shinozaki and Shinozaki, 2006; Shinozaki and Yamaguchi-Shinozaki, 2007). Therefore, current research focuses at the transcriptome, and the assumption is that up or down regulation of certain genes could explain the plant response to abiotic stresses. The amount of *Arabidopsis* microarray data available in the public domain (e.g. the data generated by the AtGenExpress consortium) is growing rapidly and is a valuable resource for research on stress tolerance. Genevestigator® is a user-friendly web-based tool that enables researchers to visualize the expression of genes from a variety of microarray experiments, including those from AtGenExpress (Zimmermann *et al*., 2005).

Genome-wide expression profiling data provide a powerful step towards a comprehensive and systemic characterization of stress responses. We performed a meta-analysis on the 150 *Arabidopsis* STGs that were identified based on data in the public domain. Analysis of the stress-related expression patterns of these STGs using the high quality *Arabidopsis* 22k microarray data in Genevestigator® showed that 40-45 % were induced by one or more abiotic stresses, while down regulation was not observed (Figure 5*A*). However, it must be understood that the expression of the other STGs can be induced under specific conditions that are not covered by the datasets represented in Genevestigator®. Hierarchical clustering of the expression values of stress-induced STGs allowed to group genes with similar expression patterns and the assumption is, based on the guilt-by-association principle, that genes within such a group may exert similar functions. Seven major clusters (cluster A-G) were obtained (Figure 5*B*) and list of the STGs within each cluster can be found in Supplementary Table S2. It can be seen that the expression of almost all STGs in cluster A, B, C and E is induced by stresses related to water deficits (cold, drought, osmotic or salt). Cluster D and G contain several STGs (e.g. HSF3, HSP101, HSFA2, MBF1C) of which the expression is induced by heat stress to increase heat tolerance. The expression of STGs in cluster F (e.g. ERD5, RAB18, ABI3) is strongly responsive to ABA and these STGs are involved in tolerance to cold, freezing and salt stress. Since 40-45 % of the *Arabidopsis* STGs are induced by stress, it can be concluded that microarray-based gene-expression profiling of stress response is a valuable approach for target gene selection which is the first step towards biotechnological applications. This is especially true for responses to dehydration, which are very well characterized, and it is becoming clear that much can be learned by genome-wide comparison of such transcriptional responses.

The importance of transcriptional networks is underscored by the fact that almost 40% of the *Arabidopsis* stress-induced STGs encode TFs (compared to ~20 % in the complete *Arabidopsis* STG list). Cluster A contains many TFs, including several NAC (e.g. ANAC019, ANAC055) TFs, zinc-finger proteins (ZAT12 and ZAT10), as well as MYB/MYC proteins. ABFs are grouped in cluster B, together with ABI1 (ABA insensitive 1) and HAB1, which are also involved in ABA responses. Important TFs in the heat responsive clusters D and G are HSF3 and SHN1, and HSF2A and MBF1C, respectively. DREB/CBF TFs are grouped in cluster E, together with COR15A of which the function during freezing tolerance is regulated by DREB/CBF TF (Jaglo-Ottosen *et al*., 1998). Cluster F contains one TF, ABI3 (ABA insensitive 3).

The stress responses of plants are controlled by both ABA-dependent and ABA-independent mechanisms (Shinozaki and Yamaguchi-Shinozaki, 2007). Genes in clusters A, D, E and G are not induced by ABA treatment at the transcriptional levels (Figure 5*B*). Indeed, many TF in these clusters, such as ZAT12 and DREB/CBF TFs, were described to be part of ABA-independent transcriptional responses to stress (Shinozaki and Yamaguchi-Shinozaki, 2007). The expression of the known ABA-dependent genes in cluster B is induced by ABA, but not as much as the ABA-induced genes from cluster F.

Since stress-induced expression is a valuable approach for the identification of STGs, we were interested in how many, yet uncharacterized, stress-induced genes could function as STG. An interesting feature in Genevestigator® is the biomarker tool, which allows to identify genes that are (co-)expressed under specific (stress) conditions. By exploring the data from the *Arabidopsis* 22k (high quality) microarray experiments, we identified genes that were induced by ABA, salt, osmotic, cold or heat treatments (Figure 6). Only genes that were induced in all the experiments related to one stress treatment were considered and this resulted in the identification of 570 stress-induced genes, of which 102 (18 %) were induced by at least two stresses. By comparison with the list of known *Arabidopsis* STGs, we could estimate that only the minority (20 %) of these genes were known STGs. Within the remaining 80% fraction, proteins with putative functions during stress tolerance (e.g. LEA-type proteins), putative master regulatory genes (TFs) and several proteins with unknown function are present. Although no concrete data are available on the actual number and identity of tested genes, it can be assumed that, since stress-induced expression was shown to be a good criterion for the identification of STGs, at least some of the remaining 80% are potential STGs and therefore excellent targets for new application towards engineering of stress tolerance. Especially the heat shock response is underexplored, with only 12 STGs for 366 heat stress-induced genes.

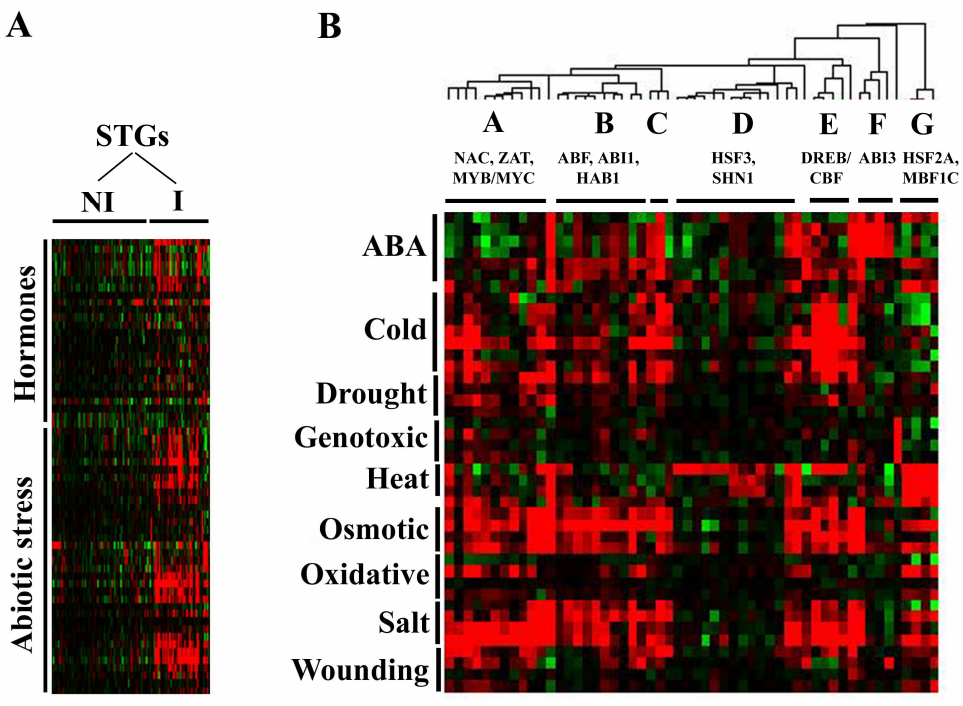

Figure 5

Clustering of the stress-related expression values of stress tolerance genes (STGs) using Genevestigator® (Zimmermann *et al*., 2005). AGI codes for approximately 150 different Arabidopsis STGs were used in the response viewer toolbox of Genevestigator® to vizualize their stress-related expression values. Genes for which no unique probeset was found were discarded. Expression of STGs in the same experiments is visualized on the horizontal axes and the expression values of one STGs across different experiments are shown vertically. Red colors indicate induction, green colors represents repressed genes. **A** Visualization of the expression values of *Arabidopsis* STGs after different hormone treatments and different abiotic stresses. Two subgroups were distinguished: STGs that are not induced by stress (NI) and genes that are stress-induced (I). **B** Detail of the subgroup containing stress-induced (I) STGs from figure 5*A*. Hierarchical clustering of the expression values after ABA treatment, and cold, drought, genotoxic, heat, osmotic, oxidative, salt and wounding is shown. For each subcluster, stress-induced TFs are shown. Multiple experiments for similar stress treatments (e.g. early and late salt stress in shoot and roots) are indicated on by vertical black bars.

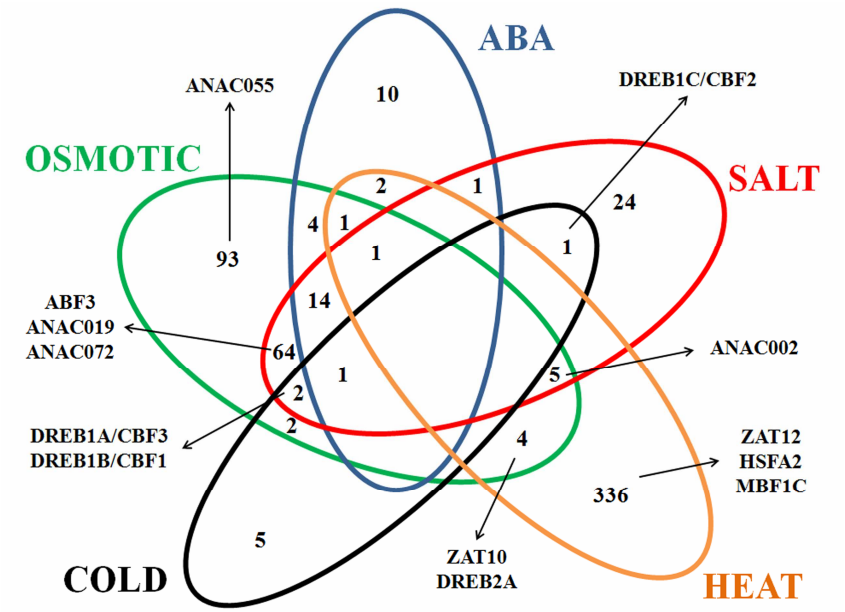

Figure 6

Stress-induced genes. Induced genes for ABA, salt, heat, cold and osmotic stress treatments were identified using the Genevestigator® biomarker tool (Zimmermann *et al.*, 2005). Only genes of which the expression was induced (ratio threshold = + 1.0, corresponding to a two-fold change increase) in all experiments for the same type of stress were retained. In the Venn diagram, numbers of genes are given that are unique to one stress or in common sections between different stresses. Empty sections indicate that no genes with matching expression profiles were found. Within specific sections, important transcription factors that have been described as stress tolerance genes (STGs) are indicated.

Molecular phenotyping of STGs to study gene function

In addition to transcriptional profiling of stress-responses, several efforts have been made to determine the molecular phenotypes of transgenic *Arabidopsis* lines perturbed in individual stress-related genes. These analyses aim to identify the downstream genes and gene networks that are affected in transgenic plants. We performed a meta-analysis on 15 published microarray datasets from stress-tolerant transgenic lines (Table 1). A current drawback that hampers a robust transcriptome meta-analysis is the diversity of the experimental platforms (either Affymetrix, Agilent or custom cDNA arrays), growth conditions, treatments, and plant developmental stages that were used to evaluate these transgenics.

Table 1. Overview of published microarrays datasets on stress-tolerant transgenic *Arabidopsis*

Gene	AGI	Stress tolerance	Construct	Cut-off	Up	Down	Reference
Affymetrix ATH1							
DREB1C / CBF2	AT4G25470	C	35S-OE	2.5 FC	152	43	Vogel *et al.,* 2005
ESK1	AT3G55990	C	KO	none	173	138	Xin *et al.,* 2007
MBF1c	AT3G24500	H, O	35S-OE	2.8 FC	167	79	Suzuki *et al.,* 2005
MKK2	AT4G29810	C, S	35S-OE	3 FC	127	25	Teige *et al.,* 2004
MYB60	AT1G08810	D	KO	2 FC	6	30	Cominelli *et al.,* 2005
XERICO	AT2G04240	D, o, s	35S-OE	2 FC	18	44	Ko *et al.,* 2006
ZAT12 (A)	AT5G59820	H, O, S	35S-OE	2.5 FC	48	158	Vogel *et al.,* 2005
ZAT12 (B)	AT5G59820	Ox	35S-OE	2 FC	102	N.D.	Rizhsky *et al.,* 2004
Agilent 22K							
ANAC072 / RD26	AT4G27410	D	35S-OE	3 FC	20	N.D.	Fujita *et al.,* 2004
AREB1 / ABF2	AT1G45249	D, H, Ox, S	35S-OE CA	3 FC	31	N.D.	Fujita *et al.,* 2005
DREB2A	AT5G05410	C, D, H	35S-OE CA	3 FC	207	N.D.	Sakuma *et al.,* 2006
HSFA2	AT2G26150	H, HL, OS, Ox, S	35S-OE	2 FC	38	9	Nishizawa *et al.,* 2006
SRK2C	AT1G78290	D, O	35S-OE	2 or 3 FC	18	14	Umezawa *et al.,* 2004
Custom							
NF-Y	AT2G38880	D, O	35S-OE	$p < 0.01$	47	60	Nelson *et al.,* 2007
DREB1D/ CBF4	AT5G51990	D	35S-OE	$p < 0.01$	167	169	Nelson *et al.,* 2007

C, cold; D, drought; H, heat; O, osmotic; Ox, oxidative; S, salt; HL, high light. Capitals indicate tolerance, lower case indicates sensitivity. FC, fold change induction of gene expression compared to control; OE, overexpression; KO, knock-out; CA, constitutive active mutation; N.D., non determined

When comparing the reported molecular phenotypes for the lines listed in Table 1, we found that the expression of none of the genes was affected in more than six transgenic lines, suggesting that stress tolerance in most of these transgenic lines is controlled by diverse molecular mechanism instead of a few common regulators. Transcript levels of in total 19 genes were induced and eight genes were downregulated in at least four out of the 15 transgenic lines (Table 2). Eight genes encoding LEA-type proteins, including several COR genes, were present in the induced gene set (~40%). Their expression is especially induced in transgenic lines with increased levels of DREB/CBF TFs and these are, at least in part, responsible for the accumulation of LEA-type proteins during cold, drought, salt and freezing stress. Promoter analysis of the COR genes indeed showed the presence of dehydration responsive elements (Yamaguchi-Shinozaki and Shinozaki, 1994). The fact that many LEA genes are simultaneously induced during stress (by one or multiple TFs), indicates that LEA-type proteins co-operate during abiotic stresses. Because of this synergistic effect, ectopic expression of a single LEA-protein is not always sufficient to confer plant tolerance, but *Arabidopsis* plants transformed with multiple LEA-type genes showed increased survival from freezing stress (Puhakainen *et al*., 2004).

Within the eight genes that were commonly repressed in at least four transgenics, disease defense genes were abundant (5 genes in total), suggesting that genes involved in tolerance to abiotic stress negatively affect tolerance to biotic stress. Such an opposite effect on abiotic and biotic stress tolerance has been recently reported for *Arabidopsis* plants expressing GLIP1 (pepper GDSL-type lipase 1), which increased tolerance to drought stress but at the same time increased susceptibility to several pathogens, and for *Arabidopsis* plants expressing HIR1 (pepper hypersensitive-induced reaction protein 1), which resulted in enhanced resistance to pathogens but increased sensitivity to drought and salt stress. (Hong *et al*., 2008; Jung *et al*., 2008). If the inverse correlation between abiotic and biotic stress tolerance is true, the engineering of plants that overcome this problem will be an important challenge for the future.

Table 2. Common induced and repressed genes in stress-tolerant transgenic *Arabidopsis*

		DREB1C/ CBF2	DREB1D/ CBF4	DREB2A	ESK1	SRK2C	HSFA2	MKK2	ABF2	RD26	ZAT12 (A)	ZAT12 (B)	XERICO	MBF1c	NF-Y	MYB60	Total[1]
Upregulated genes																	
AT2G42540	LEA-type protein, cold-regulated protein (COR15a)	484.9	17.9	13.8	6.9	10.6	2.2										6
AT2G42530	LEA-type protein, cold-regulated protein (COR15b)	53.9	15.0	3.2	3.0	3.3											5
AT5G52310	LEA-type protein, responsive to dehydration protein (RD29A) / cold-regulated protein (COR78)	57.7	10.5	12.6	2.8	4.2											5
AT1G20450	LEA-type protein, dehydrin family protein, early responsive to dehydration (ERD10)	8.6	2.6	3.7	4.3			2.5									5
AT2G23120	Unknown, similar to LEA protein	8.6	2.2	6.1	2.9			3.0									5
AT2G33380	LEA-type protein, responsive to dehydration protein (RD20)	3.1	1.7		5.5				5.5	3.3							5
AT1G58360	Amino acid permease AAP1	3.5	1.4		1.8					2.7							4
AT2G43620	Chitinase, putative; similar to glycoside hydrolase family 19 protein	21.1	4.2	3.3							6.1						4
AT5G15970	LEA-type protein, cold-regulated protein (COR6.6/KIN2)		3.6	3.1			2.2		4.8								4
AT1G09350	Galactinol synthase (GOLS3), glycosyl transferase	346.5	46.9	27.3		2.6											4
AT5G52300	LEA-type protein, responsive to dehydration protein (RD29B)	20.9		6.3	10.5				6.6								4
AT4G23600	Aminotransferase class I and II family protein, Jasmonic acid responsive 2 (JAR2)	4.6		5.9				4.5		3.6							4
AT1G72520	Lipoxygenase LOX5							5.5			5.1	2.5		4.8			4
AT4G23680	Major latex protein-related			6.3	2.9						12.6	5.3					4
AT3G28220	MATH domain-containing protein	7.8	3.5		2.7			5.0									4
AT4G17470	Palmitoyl protein thioesterase family protein				2.9						12.7	3.0	1.3				4
AT4G12490	protease inhibitor / lipid transfer protein (LTP)	3.2	2.8	3.5							13.9						4
AT1G16850	Unknown	380.5	11.2	3.7	16.6												4
AT5G57785	Unknown											2.8	1.3	3.4	1.5		4
Downregulated genes																	
AT3G22231	PATHOGEN AND CIRCADIAN CONTROLLED 1 (PCC1)	-5.7			-3.3			-3.3			-3.4				-1.4		5
AT2G26020	Plant defensin 1.2b (PDF1.2b)	-64.0	-3.1		-8.8						-29.7			-5.3			5
AT5G44420	Plant defensin 1.5 (PDF1.5)	-9.8	-3.2		-5.0						-24.5			-4.5			5
AT2G40100	Chlorophyll a/b-binding protein		-1.2			-3.5							-1.7			-2.7	4
AT3G04210	Disease resistance protein (TIR-NBS class)	-3.2	-2.7		-2.4						-5.1						4
AT5G03350	Legume lectin family protein				-2.1			-3.3			-4.5				-1.9		4
AT3G23550	MATE efflux family protein		-3.0		-3.3						-4.0		-1.1				4
AT2G14560	Unknown				-5.0			-5.0					-1.0		-2.3		4

[1] Represents the number of transgenics in which the gene is deregulated. Values indicate fold changes compared to non-transformed controls

Stress resistance: At what cost?

Increased stress resistance can be accompanied by negative effects on normal growth and development, the so-called yield penalty, and this includes growth retardation, decreased seed setting, delayed flowering, and various other negative traits depending on the plant species. In literature, a yield penalty has frequently been reported for transgenic lines with modified ABA, TF or metabolite levels. ABA is an important phytohormone that is involved in many developmental processes, such as seed germination, dormancy, and stomatal closure, hence stress-tolerant transgenic plants with altered ABA levels can have developmental problems leading to growth retardation. Growth retardation is also a common negative effect of plants with modified levels of TFs (Kasuga *et al*., 1999; Hsieh *et al*., 2002; Kang *et al*., 2002; Abe *et al.*, 2003). For example, an inverse correlation was found between the levels of DREB1A expression, the level of expression of the target gene RD29A, and plant growth (Liu *et al*., 1998). Finally, also constitutive overproduction of metabolites, such as trehalose or polyamines was shown to cause abnormalities in plants grown under normal conditions (Romero *et al*., 1997; Capell *et al*., 1998).

Negative side-effects of ectopic expression of STGs can be caused by aberrant transgene expression levels (too high expression levels, expression at the wrong time or in the wrong tissues). Such problems are inherent to the use of strong constitutive promoters such as the Cauliflower Mosaic Virus 35S promoter, which is still the most commonly used promoter in the production of transgenic *Arabidopsis* plants. For TFs, expression of the transgenes to high levels in organs and growth stages where they are normally not expressed might result in unwanted expression of target genes and consequently unwanted activation of biological processes that cause the negative growth effect. For metabolic enzymes, constitutive overexpression might be energy demanding, and therefore not optimal for plant growth.

The use of a stress-inducible promoters that control the expression level, timing, and tissue-specificity of transgene expression may be more desirable. An ideal inducible promoter should avoid gene expression in the absence of the inducing agents, and the expression of a gene that is driven by an inducible promoter should be reversible and dose-dependent. The promoters of stress-induced genes are good candidates for the identification of *cis*-regulatory elements that are recognized by specific stress-inducible TFs. For example, analysis of the promoter of the drought-induced gene RD29A revealed several *cis*-acting elements involved in its stress-induced gene expression (Yamaguchi-Shinozaki and Shinozaki, 1994).

Dedicated promoters, such *hsp*, *adh*, *rab18*, *cor15* and *rd29A*, that limit transgene expression to specific circumstances were shown to provide an excellent solution to circumvent the yield penalty. Using the stress-inducible promoter of the *rd29A* gene to increase CBF3/DREB1A

expression alleviated the growth retardation that was observed with the 35S promoter (Kasuga *et al*., 1999; Kasuga *et al*., 2004). The same *rd29A* promoter also avoided the yield penalty that was associated with constitutive expression of CBF genes in potato, without affecting the level of freezing tolerance (Pino *et al*., 2007). The yield penalty of transgenic plants with increased trehalose levels could be circumvented by using the drought-inducible *rab18* promoter (Karim *et al*., 2007). In addition to the elimination of growth defects, the use of stress-inducible promoters can increase the level of stress tolerance compared to that obtained by constitutive expression. During salt stress, yield of transgenic rice plants expressing choline oxidase (involved in glycine betaine biosynthesis) controlled by an ABA-inducible promoter was higher than that of plants in which the expression of this gene was driven by a constitutive promoter (Su *et al*., 2006). The above examples prove that stress-inducible promoters are a way forward in the genetic engineering of stress-tolerant plants.

Next generation profiling tools

Genome-wide approaches, including microarrays, are extremely valuable to analyze stress responses at an "omics" level because they allow to study the relationship between multiple genes. Affymetrix® Genechip® *Arabidopsis* TILING 1.0R arrays, which allow to visualize an additional 9000 genes compared to the ATH1 arrays, form a promising microarray platform for the future (http://www.affymetrix.com; Gregory *et al*., 2008). Especially the possibility to analyze splice variants and microRNA (miRNA) expression might be of high interest. miRNAs are small, non-coding RNAs that play critical roles in post-transcriptional gene regulation. Accumulation of miRNAs leads to breakdown or translational inhibition of endogenous mRNAs via complementary target sites. Recent evidence suggests an important role for miRNAs during abiotic stress responses in plants (Sunkar *et al*., 2007) and microarray analysis can now be used to identify miRNA of which the expression is induced by stress (Liu *et al*., 2008). Alternatively, deep sequencing can provide an effective strategy to identify stress-induced miRNA (Sunkar *et al*., 2008). An important challenge to further elucidate the (post-)transcriptional mechanisms that regulate stress tolerance in plants will be to identify the targets of stress-induced miRNAs.

Gene stacking

As tolerance to abiotic stress is a multigenic trait involving many genes at the same time, the possibility of changing the expression of multiple genes together in one plant (gene stacking) seems very attractive. Combining different genes (e.g. ABI+HAB1, *Ect*A+B+C, *Mer*A+B, *Mtl*D+*Gut*D, *Ots*A+B, GLY1+2, GSMT+DMT, MYB2+MYC2, TPS+TPP, TSI1+TSIP1, RAB18+COR47, and LTI29/ERD10+LTI30)

in one plant has previously been successful to engineer stress tolerance in plants (Abe *et al*., 2003; Garg *et al*., 2002; Ham *et al*., 2006; Jang *et al*., 2003; Karim *et al*., 2007; Miranda *et al*., 2007; Moghaieb *et al*., 2006; Puhakainen *et al*., 2004; Rai *et al*., 2006; Ruiz *et al*., 2003; Saez *et al*., 2006; Tang *et al*., 2005; Waditee *et al*., 2005). Until now, such gene combination approaches have been targeting only one metabolic (e.g. mannitol, ectoine or trehalose biosynthesis) or one signaling (e.g. ABA signal transduction) pathway. The most promising gene stacking approach to date is co-transformation of multiple genes, which has many advantages over conventional procedures such as crossing and re-transformation (Halpin, 2005). Vectors for co-transformation of multiple transgenes by sequential rounds of Gateway recombination cloning (MultiRound Gateway) are currently being developed and implemented (Chen *et al*., 2006; Karimi, personal communication). A major challenge will be to change multiple biological pathways together in one plant in order to increase tolerance to various environmental stresses.

Translational biology: Using information from *Arabidopsis* to engineer stress tolerant crops

World food and feed security is increasingly dependent on continuous crop improvement and, in particular, the development of crops with increased stress resistance. An important interest of many plant biologists working with *Arabidopsis* is not only better understanding of *Arabidopsis* growth and development, but also how to exploit this knowledge to improve stress tolerance of agricultural crops (Zhang *et al*., 2004). An extensive list of reported transgenic crops with increased tolerance to abiotic stress is shown in Supplementary Table S3.

Basically, the strategies used to increase stress tolerance in *Arabidopsis* also work in crops. A attractive approach has been to express an *Arabidopsis* STG with known function during stress tolerance in a crop species of interest (Table 3). Such an approach has been successful for almost all of the described functional classes, including signaling (TFs, protein kinases), ion transport, molecular protection, osmoprotection. Alternatively, several crop orthologues of known *Arabidopsis* STGs have been studied during stress tolerance in crops. Especially well-characterized are crop orthologues of ion transport proteins, such as NHX1, and CBF/DREB1 and DREB2 TFs (Agarwal *et al*., 2006; Nakashima and Yamaguchi-Shinozaki, 2005; Yamaguchi and Blumwald, 2005; Zhang *et al*., 2004). As several tolerance mechanisms are conserved between *Arabidopsis* and crops, it can be expected that *Arabidopsis* will continue to be an excellent model, both as experimental system and as gene source, to study the abiotic stress response of plants, which will eventually lead to applications in crop biotechnology.

A number of limiting factors should be considered when translating results from *Arabidopsis* in crops (Vinocur and Altman, 2005; Yamaguchi and Blumwald, 2005). Most studies in *Arabidopsis*

focus on short-term stress treatments to evaluate the stress tolerance against high stress doses. From an agronomical point of view, it is more interesting to study the stress effects on plant growth and yield over longer periods that mimic more the life span of most crops. Another limitation is that stress responses are usually studied in laboratory conditions which do not reflect natural conditions in the field, where cycles of stress and recovery from stress are more prevalent. Moreover, plants in the field can be exposed to series of different stresses or combinations of multiple stresses at the same time. For the above reason, tolerance mechanism to one stress should always be assessed with respect to its cross-talk with other stresses.

Table 3. Stress-tolerant transgenic crops by using *Arabidopsis* genes

Gene	Molecular Function	Crop Species	Stress Tolerance	References
CBF3 / DREB1A	Transcription Factor (Cold binding factor, Drought-Responsive Element Binding protein)	Potato / Rice / Wheat	D, S and C/F	Pino *et al.,*2007 / Oh *et al.,*2005 / Pellegrineschi *et al.,*2004
HRD/HARDY	Transcription Factor, AP2/ERF-like	Rice	D and S	Karaba *et al.,*2007
P5CR	Pyrroline carboxylate reductase (proline accumulation)	Soybean	D, S and H	Kocsy *et al.,*2005 / De Ronde *et al.,* 2001/2004
PARP1	Poly(ADP-ribose) polymerase	Rapeseed	D, H and Ox	Block *et al.,*2005
PARP2	Poly(ADP-ribose) polymerase	Rapeseed	D, H and Ox	Block *et al.,*2005
ABF3	Transcription Factor (binds ABA responsive elements)	Rice	D	Oh *et al.,*2005
FTB/ERA1	Farnesyltransferase	Rapeseed	D	Wang *et al.,*2005
NDPK2	NDP kinase 2	Potato	H and Ox	Tang *et al.,*2007
HSP101	Heat shock protein	Rice	H	Katiyar-Agarwal *et al.*, 2003
CBF1 / DREB1B	Transcription Factor (Cold binding factor, Drought-Responsive Element Binding protein)	Potato / Tomato	C/F	Pino *et al.,* 2007 / Hsieh *et al.,* 2002
GPAT	Glycerol-3-phosphate acyltransferase of chloroplasts	Rice	C/F	Ariizumi *et al.,*2002
NHX1	Vacuolar Na+/H+ antiporter	Cotton / Wheat / Rapeseed	S	He *et al.,*2005 / Xue *et al.,*2004 / Zhang *et al.,*2001
P5CS	Pyrroline carboxylate synthase	Potato	S	Hmida-Sayari *et al.,*2005
MT2a	Metallothionein	Broad bean	M	Lee *et al.,*2004
MT3	Metallothionein	Broad bean	M	Lee *et al.,*2004

C, cold; D, drought; F, freezing H, heat; OS, osmotic; OX, oxidative; S, salt; M, heavy metals

CONCLUSIONS AND PERSPECTIVES

Transcriptome analysis have been effectively used to study the response of plants exposed to abiotic stress, as well as the molecular mechanisms underpinning tolerance in stress-resistant transgenic lines, and such studies have contributed significantly to the identification of genes that enhance stress tolerance when engineered in plants. *Arabidopsis* has proven to be a good model system to analyze stress tolerance mechanisms in plants, with many successful applications in crop species. New examples from early 2008 already indicate that *Arabidopsis* and microarray-based approaches will continue to dominate abiotic stress research (Yoshida *et al*., 2008; Weston *et al.,* 2008).

In addition to transcriptome profiling, profiling of the metabolome now offers an important tool to study the metabolic adjustments that occur during stress (Rizhsky *et al*., 2004; Seki *et al*., 2007) and this will, together with proteome data, contribute to enhance our knowledge on the response of plants during stress conditions. Systems biology approaches relying on the integration of such "omics"-based data will most certainly help to better understand the response of plants to abiotic stress. A major challenge for the future will be to implement all the various data to engineer well-adapted plants that produce the required high amount of biomass under both stress and non-stressed conditions.

Supplementary Tables

Supplementary Tables for this chapter are included as addendum at the back of this thesis and are also made available in an electronic version on the attached compact disc.

Supplementary Table S1. *Arabidopsis* stress tolerance genes

Supplementary Table S2. Stress-related expression clusters of the *Arabidopsis* stress tolerance genes

Supplementary Table S3. Stress-tolerant transgenic crop species

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I have attempted to include all of the most relevant findings concerning transgenic plants with increased stress tolerance in Supplementary Tables S1 and S3, but due to the rapid progress in this area of research, certain findings may have been left out and I apologize for that.

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PART II

HYDROGEN PEROXIDE SIGNALING IN PLANTS

CHAPTER 2

Hydrogen peroxide-induced gene expression across kingdoms: A comparative analysis

ABSTRACT

Cells react to oxidative stress conditions by launching a defense response through the induction of nuclear gene expression. The advent of microarray technologies allowed monitoring of oxidative stress-dependent changes of transcript levels at a comprehensive and genome-wide scale, resulting in a series of inventories of differentially expressed genes in different organisms. We performed a meta-analysis on hydrogen peroxide (H_2O_2)-induced gene expression in the cyanobacterium *Synechocystis* PCC 6803, the yeast *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, the land plant *Arabidopsis thaliana*, and the human HeLa cell line. The H_2O_2-induced gene expression in both yeast species was highly conserved and more similar to the *A. thaliana* response than that of the human cell line. Based on the expression characteristics of genuine antioxidant genes, we show that the antioxidant capacity of microorganisms and higher eukaryotes is differentially regulated. Four families of evolutionarily conserved eukaryotic proteins could be identified that were H_2O_2-responsive across kingdoms: DNAJ domain-containing heat shock proteins, small GTP-binding proteins, Ca^{2+}-dependent protein kinases, and ubiquitin-conjugating enzymes.

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INTRODUCTION

All aerobic organisms frequently experience endogenous and environmental conditions that provoke the accumulation of reactive oxygen species (ROS). Hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), and singlet oxygen are highly reactive molecules and, therefore, potentially harmful at higher concentrations. They can haphazardly assault proteins, lipids, DNA and any other cellular component, thereby causing severe damage. Consequently, aerobic organisms have developed or adapted an efficient ROS-scavenging machinery, involving enzymes such as superoxide dismutases (SODs) and catalases together with an extensive battery of non-enzymatic antioxidants (Halliwell 2006).

Compared to other ROS, H_2O_2 is a relatively long-lived molecule (one millisecond) that is able to diffuse across cell membranes (Bienert *et al*., 2006). This characteristic is compatible with its role as a signaling molecule during growth and development (Finkel and Holbrook 2000; Sauer *et al*., 2001; Neill *et al*., 2002; Van Breusegem and Dat 2006). The transduction of H_2O_2 signals into biologically relevant information is governed by sensors or receptors, mitogen-activated protein kinases, and transcription factors and has been suggested to be evolutionarily conserved (Toone and Jones 1998; Georgiou 2002; Liu *et al*., 2005). The best known example is the ASK1/JNK (apoptosis signal-regulating kinase 1 / c-jun N-terminal kinase) cascade that activates the AP-1 transcription factor through the oxidation of cysteine residues (Abate *et al*., 1990; Delauney *et al*., 2000; Shen *et al*., 2006). In yeast, different H_2O_2 levels trigger independent signaling pathways (Vivancos *et al*., 2006). H_2O_2-signaling in plants is coordinated via a complex network that involves multiple protein kinases and transcription factors (Mittler *et al.,* 2004; Miller and Mittler, 2006; Kaminaka *et al.,* 2006). The coordinated action of two redox-regulated transcription factors, TGA1 (TGACG motif-binding factor 1) and NPR1 (non expressor of pathogenesis-related genes 1), is required for defense gene expression and systemic acquired disease resistance in *Arabidopsis thaliana* (Després *et al*., 2003; Mou *et al*., 2003).

Since recently, transcriptional changes can be monitored on a genome-wide scale by using different technologies, such as differential display, expressed sequence tag sequencing, serial analysis of gene expression, cDNA-amplified fragment length polymorphism, microarrays and deep sequencing technologies (Lockhart and Winzeler 2000; Donson *et al*., 2002; Vandenabeele *et al*., 2003; Emrich *et al*., 2007). Such analysis has led to comprehensive inventories of genome-wide H_2O_2-related gene expression in bacteria (*Escherichia coli* and *Bacillus subtilis*), the cyanobacterium *Synechocystis* sp. strain PCC 6803, two yeast species (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*), mouse (*Mus musculus*), fruitfly (*Drosophila melanogaster*), several human (*Homo sapiens*) cell lines, and one plant species (*Arabidopsis thaliana*) (Gasch *et al*., 2000;

Zheng *et al*., 2001; Chuang *et al*., 2002; Chen *et al*., 2003; Lee *et al*., 2003; Desaint *et al*., 2004; Girardot *et al*., 2004; Kobayashi *et al*., 2004; Li *et al*., 2004; Mostertz *et al*., 2004; Murray *et al*., 2004; Davletova *et al*., 2005; Kim *et al*., 2005; Vanderauwera *et al*., 2005). These gene expression studies clearly showed that increased cellular H_2O_2 levels have a considerable impact on the transcriptome of all species, by changing the expression of hundreds of genes. H_2O_2 not only affects genes involved in ROS detoxification, but also drives the expression of genes involved in signal transduction, transcriptional regulation and protein, carbohydrate or lipid metabolism, illustrating the complexity of the transcriptional response to H_2O_2.

We present a comparative transcriptome analysis that assesses, at a genome-wide scale, the similarity of the H_2O_2-dependent transcriptional response in evolutionarily distant species. Besides some species or lineage-specific H_2O_2 responses, our analysis identified a confined set of similarly induced gene products in eukaryotes, with a strong conservation in yeast and *Arabidopsis*.

RESULTS AND DISCUSSION

Identification of Homologous Gene Products across Kingdoms

We compared H_2O_2-driven gene expression in evolutionarily distant species by performing a meta-analysis on publicly available microarray data sets from five completely sequenced and annotated species (*Synechocystis* PCC 6803, *S. cerevisiae*, *S. pombe*, *H. sapiens* and *A. thaliana*). These five species were selected because the relevant microarray studies were available and, with the exception of the protista, they cross the different biological kingdoms, hence spanning a broad evolutionary distance (Margulis 1992; Hedges and Kumar 2003).

A first necessary step was the identification of homologous gene products within the different organisms. Therefore, we clustered the protein sequences of the different species with TRIBE-Markov Clustering (TRIBE-MCL). This algorithm allows a fast and accurate classification of large protein data sets into protein families and has multiple advantages over alternative protein clustering methods (Enright *et al*., 2002). Protein sequences of a second plant (*O. sativa*) and mammalian species (*M. musculus*) were included to improve the clustering outcome.

The clustering resulted in 16,207 protein families (containing more than one protein) encompassing 118,020 individual proteins in total. The different protein families were first evaluated according to size (protein number), species number, and species representation. Family size was opposite proportional to frequency of occurrence, with the majority (>95%) of all families smaller than 20 proteins (Figure 1*A*). Most protein families were restricted to one (3232; 20%) or two (9112; 56%) species, reflecting the large evolutionary distances between the species (Figure

1*B*). The species representation of all 16,207 protein families pointed to the existence of a limited set of highly conserved proteins (belonging to 244 protein families) that have a molecular function similar in *Synechocystis* and in eukaryotes, as well as to a substantial diversity among the different kingdoms (Figure 2). In addition, with this analysis, 1,244 conserved eukaryotic protein families were identified (Figure 2).

Quality of the TRIBE-MCL clustering was further assessed by manual inspection of the phylogenetic profiles of several genuine antioxidant enzymes: SODs, catalases, and peroxiredoxins, all known to have an evolutionarily conserved function during ROS detoxification (Touati 1988; Zamocký and Koller 1999; Rhee *et al*., 2005). As expected, protein sequences of SODs, catalases and peroxiredoxins were contained within specific protein families (data not shown). Proteins with abundant domains, including protein kinase or DNA-binding domains were frequently found in larger, more divergent protein families containing more than 200 proteins (Riechmann *et al*., 2000; Wang *et al*., 2003). Because TRIBE-MCL correctly grouped proteins with significant sequence similarity and almost identical functions, we concluded that the clustering in protein families was accurate and reliable for further analysis.

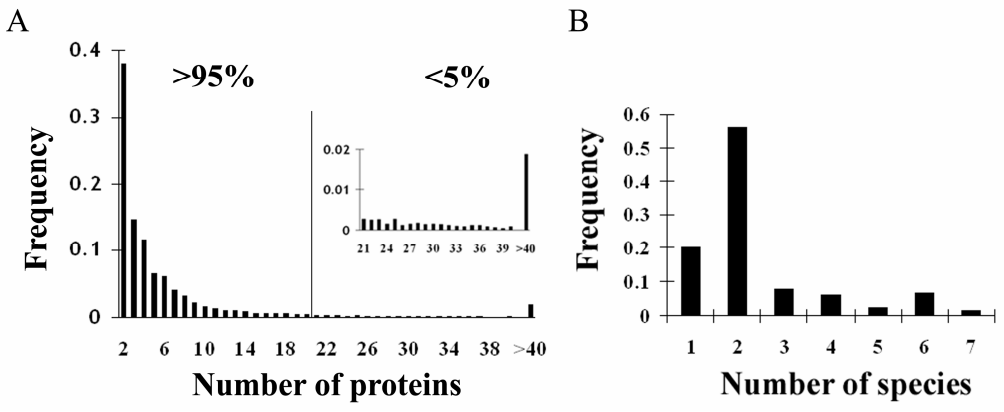

Figure 1

Frequency distribution of protein family size and species number for 16,207 protein families. **A** Histogram of protein family size, represented as protein number. The distribution of protein families with more than 20 proteins is blown up in the inset image. For sake of clarity, protein families containing more than 40 proteins (frequency < 0.001) are not shown separately. **B** Frequency distribution of species number.

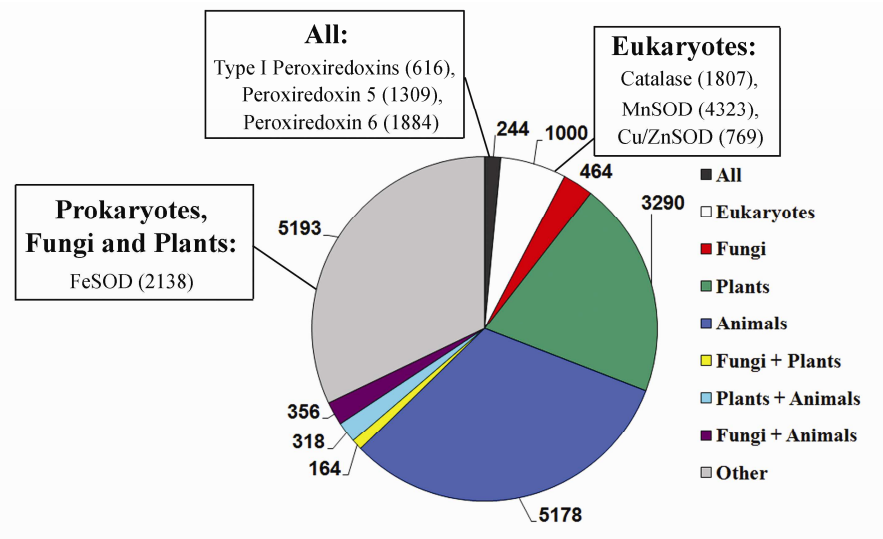

Figure 2

Evolutionary distribution of all protein families. *All* include protein families with representatives in all analyzed species. *Eukaryotes* group protein families with representatives in all species other than *Synechocystis*. *Fungi*, *Plants*, and *Animals* include protein families with only representatives for fungi, plants, and animals, respectively. The total number of protein families in each category is indicated. The evolutionary conservation of different SODs, catalases, and peroxiredoxin protein families is boxed. Family ID numbers are shown in parentheses.

H_2O_2-induced gene expression in evolutionarily distant species

For prokaryotes, we selected a microarray experiment that followed the expression of 3168 genes from *Synechocystis* sp. strain PCC 6803 after addition of 1.5 mM H_2O_2 to a cell culture (Li *et al*., 2004). In the yeast experiments, cDNA microarrays (containing approximately 5200 and 6000 *S. pombe* and *S. cerevisiae* genes, respectively) were used to monitor gene expression after addition of H_2O_2 (0.3-0.5 mM) to a cell culture (Gasch *et al*., 2000; Chen *et al*., 2003). To avoid an additional level of complexity related to tissue-specific responses in multicellular organisms, single-cell systems were also used to study H_2O_2-induced stress responses in animals and plants. For *H. sapiens*, we selected a microarray analysis of 25,802 genes in HeLa cells treated with different H_2O_2 concentrations (Murray *et al*., 2004). For the plant kingdom, we opted for an experiment in which microarrays (representing 25,636 genes) were used to monitor the transcriptional changes of 2-week-old, liquid-cultured *A. thaliana* seedlings that were treated with 5 mM H_2O_2 (Kim *et al*., 2005). More details on the selected microarray experiments can be found in Table 1. Due to the heterogeneity of the experimental setups, we used relative expression data to identify differentially expressed genes. Figure 3 presents the kinetics of the transcription response, showing the number of genes with 2-, 3-, 4-, and more than 5-fold changes within these experiments. In all species, a significant up- and down-regulation of transcript levels occurred, but we focused only on the

inductive response, because, in most cases, it starts earlier than the repressed response, enabling one to target upstream genes with minimal interference of secondary effects. Within the early time points, we selected those at which the strongest induction was observed: 30 min, 30 min, 1 h, 6 h, and 3 h for *Synechocystis*, *S. cerevisiae*, *S. pombe*, human HeLa cell lines, and *A. thaliana*, respectively. Genes with an H_2O_2-induced expression of at least two-fold were retained for further analysis (161, 607, 578, 298, and 690 genes for *Synechocystis*, *S. cerevisiae*, *S. pombe*, human HeLa cell line, and *A. thaliana*, respectively).

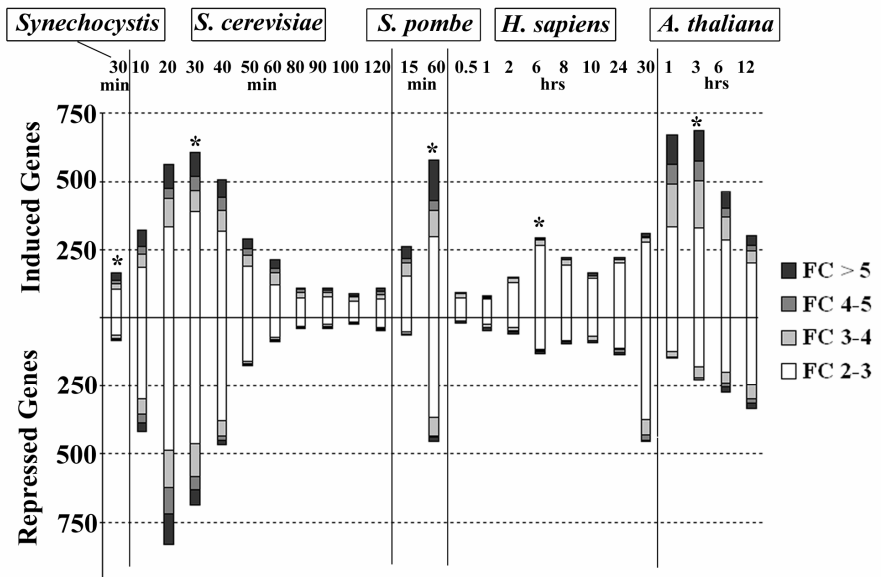

Figure 3

Kinetics of the transcriptional response within the individual ROS experiments. The number of transcripts with a 2 to 3, 3 to 4, 4 to 5 and >5-fold increase or decrease in expression at the different time points are represented by white, light grey, dark grey, and black bars, respectively. Time points indicated with asterisks were used for this study. FC, Fold Change.

H_2O_2 response matrix as a tool for comparing gene expression

First, we identified H_2O_2-responsive protein families. Within a species, a protein family was considered to be responsive when at least one of its members was more than twofold upregulated. A H_2O_2 response matrix was obtained that indicated how many gene products within each family were responsive. The data can be queried on our website (http://bioinformatics.psb.ugent.be/supplementary_data/strob/h2o2/). The number of H_2O_2-responsive families varied for each species and were proportional to the total number of H_2O_2-responsive genes. In *S. cerevisiae*, *S. pombe*, and *A. thaliana*, approximately 400 families were

responsive to H_2O_2, in contrast to only 101 and 168 families in *Synechocystis* and *H. sapiens,* respectively (Table 1). We used the H_2O_2 response matrix to determine the evolutionary conservation of the H_2O_2 response in all species (Figure 4*A*). The H_2O_2 responsiveness of 87% of the 1253 families was restricted to one species, indicating a strong species-specific response (Figure 4*B*). Table 2 presents the pairwise overlap of the H_2O_2 response between the different species. Not surprisingly, the overlap was the largest between *S. cerevisiae* and *S. pombe* with 107 common H_2O_2-responsive protein families (p-value < 0.001), revealing that the H_2O_2-induced transcriptional response is highly conserved between these two yeasts. Our data, together with the conserved core environmental stress responses of distant yeast species, indicate that stress responses in general are well conserved in yeast (Chen *et al*., 2003).

Table 1. Overview and details of the five selected microarray experiments

Species	Microarray platform	Genes represented	Treatment H_2O_2 (mM)	Time points	Induced genes[b]	Responsive protein families	Reference
Synechocystis	GST[a]	3168	1.5	30 min	121	101	Li *et al*., 2004
S. cerevisiae	GST[a]	6000	0.30	10, 20, 30, 40, 50, 60, 80, 90, 100, 120 min	504	403	Gasch *et al.*, 2000
S. pombe	GST[a]	5269	0.50	15, 60 min	504	392	Chen *et al*., 2003
H. sapiens HeLa	cDNA	25802	0.60	0.5, 1, 2, 8, 16, 24, 30 h	191	168	Murray *et al.,* 2004
A. thaliana	GST[a]	25636	5	1, 3, 6, 12 h	658	390	Kim *et al*., 2005

[a] GST, Gene Specific Tag; [b] H_2O_2-induced genes with homologous gene products

Table 2. Pairwise comparisons of H_2O_2-induced transcriptional responses

Species	*Synechocystis*	*S. cerevisiae*	*S. pombe*	*H. sapiens*	*A. thaliana*
Synechocystis		407	412	446	867
S. cerevisiae	9		2476	1961	1757
S. pombe	7	107[*]		2067	1864
H. sapiens	1	13	10		1981
A. thaliana	8	40	44	10	

Above and under diagonal, numbers of protein families with genes from both species and observed numbers of common H_2O_2-induced protein families, respectively. [*] $p < 0.001$

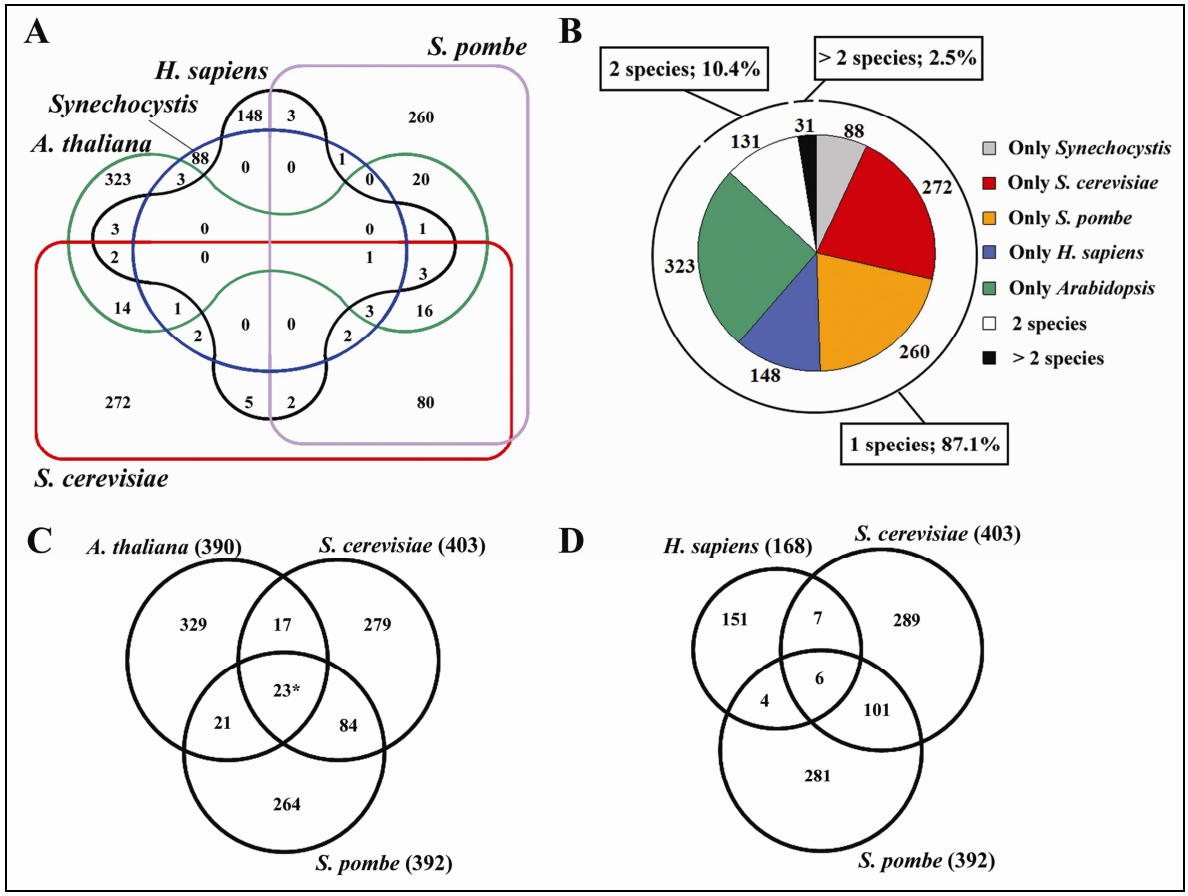

Figure 4

Evolutionary conservation of the H_2O_2-inductive response. Each value represents a number of responsive protein families. **A** Venn diagram illustrating the conservation between *Synechocystis*, *S. cerevisiae*, *S. pombe*, *H. sapiens*, and *A. thaliana*. **B** Pie diagram showing the number of families of which the response is conserved in only one species, in just two species, and more than two species. **C** Detailed Venn diagram demonstrating the overlap between *S. cerevisiae*, *S. pombe* and *A. thaliana*. **D** Detailed Venn diagram showing the conservation in *S. cerevisiae*, *S. pombe*, and *H. sapiens*. * $p < 0.05$.

Four protein families within the core eukaryotic H_2O_2 response

In addition to protein families that were H_2O_2 responsive in only two species, 31 protein families were responsive in at least three species (Table 3). Remarkably, 23 families were responsive in *A. thaliana*, *S. cerevisiae*, and *S. pombe*, but only six families were responsive in *H. sapiens* and both yeasts (Figure 4*C* and 4*D*). Although the low number of H_2O_2-responsive families in *H. sapiens* is partially responsible for this difference, the conservation between both yeasts and *A. thaliana* was significant (*p*-value < 0.05) and that with *H. sapiens* was not. These data demonstrate that the transcriptional response to increased H_2O_2 levels in yeast is more similar to that of plants than to that of animals.

Table 3. Protein families (31) with a conserved H_2O_2 expression profile, shown as fraction of H_2O_2-induced genes, in at least three species

Family ID	Total entries	Family description	*Synechocystis*	*S. cereviase*	*S. pombe*	*A. thaliana*	*H. sapiens*
All species							
38	197	DNAJ heat shock protein	0.40	0.19	0.11	0.05	0.03
All eukaryotes							
8	361	Ras-related GTP binding protein	N.R.	0.05	0.17	0.05	0.04
44	184	Ca-dependent (S/T) protein kinase	N.R.	0.25	0.40	0.02	0.03
47	176	Ubiquitin-conjugating enzyme	N.R.	0.08	0.15	0.05	0.03
Unicellular organisms							
87	108	Short chain dehydrogenase /(oxido)reductases	0.12	0.20	0.50	0.00	0.00
616	25	Thioredoxin peroxidase (Type II peroxiredoxins)	0.25	0.67	0.67	0.00	0.00
All species-*H. sapiens*							
28	231	ATPase, AAA family /FtsH protease	0.33	0.19	0.05	0.02	0.00
182	63	Small HSPs	1.00	1.00	0.50	0.24	N.R.
578	26	D-3-phosphoglycerate dehydrogenase	0.33	0.20	0.40	0.20	0.00
Yeast and *A. thaliana*							
13	329	Protein Kinase/MAPK	N.R.	0.23	0.05	0.01	0.00
15	295	(serine/threonine) protein kinase	0.00	0.23	0.22	0.04	0.00
20	259	Zinc Finger, C3HC4 type (RING finger)	N.R.	0.50	0.50	0.06	N.R.
41	195	EF hand Ca/Cal binding protein	N.R.	0.50	0.17	0.11	0.00
58	156	ABC transporter, subfamily G	0.00	0.10	1.00	0.02	0.00
130	80	Cinnamoyl-CoA /anthocyanidin reductase	0.00	0.25	1.00	0.12	NR
148	72	ABC transporter, subfamily C	0.00	0.14	0.25	0.19	0.00
151	72	Oxidoreductase, alcohol (aldo/keto) reductase family	0.00	1.00	0.83	0.20	0.00
154	71	Cation-transporting ATPase	0.00	0.20	0.33	0.20	0.00
199	60	Glutaredoxin	0.00	0.75	0.33	0.04	0.00
361	36	Trehalose-phosphatase/ glycosyl transferase	0.00	0.75	0.60	0.09	N.R.
363	36	Oxidoreductase, zinc/NADP-dependent dehydrogenase	N.R.	1.00	1.00	0.43	0.00
382	35	Heavy metal-transporting ATPase	0.00	0.50	1.00	0.12	0.00
1356	14	GTP cyclohydrolase	0.00	0.50	0.33	0.20	N.R.
1541	13	Ribonucleoside-diphosphate reductase	0.00	0.50	1.00	0.33	0.00
1818	11	Gluthatione peroxidase	0.00	0.67	1.00	0.25	N.R.
Yeast and *H. sapiens*							
1	1093	Zinc Finger protein	N.R.	0.14	0.27	0.00	0.001
179	64	Ubiquitin-protein ligase	N.R.	0.25	0.33	0.00	0.05
Other[a]							
27	235	Serine/threonine-protein kinase/MAPKKK	N.R.	0.00	0.06	0.02	0.04
55	165	Sugar Transporter	0.00	0.17	0.00	0.04	0.13
331	38	Transcription Factor/Jumonji/ARID domain-containing protein	N.R.	0.50	0.00	0.10	0.20
311	40	HSP 90	1.00	0.50	0.00	0.12	0.00

[a] Include protein families with a conserved H_2O_2 response in any combination of three species that is not represented in the other categories. ABC, ATP-binding cassette; ARID, adenine/thymine-rich interaction domain; EF, α-helices E and F of parvalbumin; NADP, nicotinamide adenine dinucleotide phosphate; N.R., no representative protein found. RING, really interesting new gene

Three protein families (representing GTP-binding proteins, protein kinases, or ubiquitin (Ub)-conjugating enzymes) were induced in yeast, *A. thaliana* and *H. sapiens*, but had no homologs in the prokaryote *Synechocystis*, restricting this conservation to eukaryotes. One protein family, representing DNAJ heat shock proteins (HSPs), was induced by H_2O_2 in all kingdoms. Together, these four protein families were defined as the "core eukaryotic H_2O_2 response". In the remaining protein families no conserved response was found within one of the above mentioned species combinations.

It is known that HSPs, GTP-binding proteins, protein kinases, and Ub-conjugating enzymes function in evolutionarily conserved biological processes, such as heat shock response, cellular signaling, or protein metabolism. Therefore, they might also have an important and conserved role in responses to oxidative stimuli. This analysis suggests that this conserved functionality requires, at least to some extent, regulation at the transcriptional level.

Evolutionarily conserved H_2O_2-induced heat shock response

The proteins with the best evolutionarily conserved response to H_2O_2 are DNAJ HSPs, which are molecular chaperones defined by the presence of the conserved J domain (Table 3). They can stimulate the substrate-binding activity of 70-kDa HSPs, thereby modulating accurate protein folding and transport (Walsh *et al*., 2004). Other HSPs (HSP90 and HSP20) were also, albeit less conserved, induced by H_2O_2 (Table 3). In addition, H_2O_2-induction of HSPs has been reported in other species, such as tomato, rice and *Drosophila* (Courgeon *et al*., 1990; Banzet *et al*., 1998; Lee *et al*., 2000). The conserved need for HSPs during oxidative stress might be explained by the chaperone function that HSPs can exert on oxidatively damaged and partially denatured proteins (Jakob *et al*., 1999). Alternatively, heat shock factors can act as direct sensors of H_2O_2, thereby regulating the expression of defense genes and subsequent protection during oxidative stress (Ahn and Thiele 2003; Volkov *et al*., 2006; Miller and Mittler 2006). Because of their protective function, loss of HSPs leads to increased sensitivity, while constitutive expression of some HSPs (such as chloroplastic HSP21) enhances the tolerance toward heat and H_2O_2 stress (Härndahl *et al*., 1999; Jacob *et al*., 1999; Ahn and Thiele, 2003; Neta-Sharir *et al*., 2005). Together, these observations suggest a significant overlap between the heat shock and oxidative stress response in all kingdoms.

Eukaryotic H_2O_2 signaling involves induction of G-proteins and Ca^{2+}-dependent protein kinases

We observed a conserved H_2O_2 induction in *S. pombe*, *S. cervisiae*, *A. thaliana* and *H. sapiens*, for one family of small, ras-like GTP-binding proteins (G proteins), and one protein family containing calcium (Ca^{2+})-dependent protein kinases (Table 3). Both ras-like G-proteins and protein kinases

have already been implicated in oxidative stress signaling in yeast, plants, and mammals, suggesting a conserved function for such proteins (Toone and Jones, 1998; Finkel and Holbrook, 2000; Essers *et al*., 2004; Rentel *et al*., 2004). Closer investigation of the G-protein family revealed that it represents Rab GTP-binding proteins, one of the five subfamilies of ras-like GTPases (Vernoud *et al*., 2003). Rab GTPases are mainly involved in cellular trafficking, but at least in plants, they might have evolved additional functions (Rutherford and Moore, 2002). However, a role for Rab proteins during oxidative stress signaling has not been elucidated yet.

Environmental or cellular stimuli, including oxidative stress, can cause changes in calcium (Ca^{2+}) patterns, which can be sensed by specific Ca^{2+}-dependent protein kinases and decoded into downstream effects, such as altered protein phosphorylation and gene expression (Cheng *et al*., 2002). In animals, it is well known that H_2O_2 can activate Ca^{2+}-dependent protein kinases to prevent oxidative stress-induced cell death (Franklin *et al*., 2006).

In addition to the importance of G-proteins and Ca^{2+}-dependent protein kinases in controlling the eukaryotic response to oxidative stress, our data suggest the involvement of transcriptional regulation of these genes by H_2O_2, which might be essential for signal amplification and cross-talk during oxidative stress. This hypothesis would be in agreement with the general function of ras-like G-proteins and protein kinases in multiple, interconnected signaling cascades that control various biological processes (Matozaki *et al*., 2000).

Conserved H_2O_2-induced Ubiquitination Response in Eukaryotes

Ub-conjugating enzymes act within proteasome-dependent proteolysis where they transfer Ub molecules, either directly or via an Ub ligase, to a substrate protein, a process known as ubiquitination (Pickart 2001). Transcripts of Ub-conjugating enzymes were induced by H_2O_2 in all four eukaryotes (Table 3). A robust ubiquitination response and a transient increase in activity of the Ub-dependent pathway has been demonstrated to occur in lens cells exposed to oxidative stress, resulting in enhanced recovery after oxidative stress (Shang *et al*., 1997). This protection is probably a result of the targeted removal of oxidized or damaged proteins by Ub-conjugating enzymes, or the Ub-dependent proteolytic pathway in general (Shang *et al*., 1995). The importance of the Ub-dependent pathway during oxidative stress is further highlighted by the requirement of a functional polyubiquitin gene to withstand toxic H_2O_2 levels in yeast (Cheng *et al*., 1994).

A conserved antioxidant response in unicellular organisms

Besides four protein families with a conserved H_2O_2 induction in eukaryotes, we also observed a significant (*p*-value < 0.05) conservation within all the unicellular organisms, with two families showing a specific transcriptional induction in *Synechocystis*, *S. cerevisiae* and *S. pombe*: short-chain dehydrogenases/reductases (SDR) and type-I peroxiredoxins (Table 3). Both SDR and peroxiredoxins are evolutionarily conserved proteins that are directly involved in the protection of cells against oxidative stress (Rhee *et al*., 2005; Kallberg *et al*., 2002). For example, constitutive expression of a SDR protein confers protection against oxidative stress-induced cell death via the detoxification of highly reactive xenobiotics (Botella *et al*., 2004). Peroxiredoxins are thioredoxin-dependent peroxidases that remove H_2O_2 and peroxinitrites (Rhee *et al*., 2005). The importance of peroxiredoxins as antioxidants is further illustrated by their capacity to prevent H_2O_2-induced apoptosis in human cells (Yuan *et al*., 2004).

In addition to SDR and type I peroxiredoxins, H_2O_2-induction of genuine antioxidant enzymes, such as catalases, SODs, glutathione peroxidases, ascorbate peroxidases, and type II peroxiredoxins, was also restricted to unicellular organisms (Table 4). Catalases, Cu/ZnSODs and glutathione peroxidases were induced in both yeast species, but not in *Synechocystis*. Other antioxidant enzymes showed less conserved expression patterns in unicellular organisms. However, none of the antioxidant genes were induced by H_2O_2 in *A. thaliana* and *H. sapiens*. These data indicate that unicellular antioxidant systems are part of the oxidative stress-inducible adaptive responses, while higher eukaryotes carry a rather constitutive transcriptional antioxidant response during H_2O_2-induced oxidative stress (Storz and Imlay 1999). Although transcriptional control of antioxidants genes in specific (oxidative) stress situations cannot be excluded, antioxidant gene expression of animals and plants seems to be controlled at the posttranscriptional level. MnSOD production in animal models, for example, is regulated via the binding of an unidentified MnSOD mRNA-binding molecule (Clerch *et al*., 2000). In plants, posttranscriptional regulation of ascorbate peroxidase levels has been evidenced during programmed cell death and drought stress (Mittler *et al*., 1998; Mittler and Zilinskas 1994). Recently, a microRNA molecule (miR398) has been identified as a repressor of Cu/ZnSOD expression in *A. thaliana* and downregulation of miR398 is important for tolerance against oxidative stress (Sunkar *et al*., 2006). These data suggest that post-transcriptional control of antioxidant gene expression might be very important in mammals and plants.

Table 4. H_2O_2 responsiveness of antioxidant genes, shown as fraction of H_2O_2-induced genes

Family ID	Family description	*Synechocystis*	*S. cerevisiae*	*S. pombe*	*A. thaliana*	*H. sapiens*
1807	CAT	N.R.	0.50	1.00	0.00	0.00
2138	FeSOD	0.00	0.00	0.00	0.00	N.R.
4323	MnSOD	N.R.	1.00	0.00	0.00	0.00
769	Cu/ZnSOD	N.R.	0.50	0.50	0.00	0.00
413	GPX	0.00	1.00	1.00	0.00	0.00
812	APX/CAT peroxidase	0.00	1.00	N.R.	0.00	N.R.
1309	Peroxiredoxin 5 (Type 2)	1.00	1.00	0.00	0.00	0.00
1884	Peroxiredoxin 6 (Type 2)	0.00	1.00	N.R.	0.00	0.00
616	Peroxiredoxin 1-4 (Type 1)	0.25	0.67	0.67	0.00	0.00

APX; ascorbate peroxidase; CAT, catalase; GPX, glutathione peroxidase; N.R., no representative protein found.

Conserved H_2O_2-Induction of Protein Families with Unknown Function

To investigate the H_2O_2 response of genes with unknown function, we manually analyzed our set of 162 different protein families that were responsive in at least two species for the overrepresentation of unknown, expressed, or hypothetical proteins. Because most unknown proteins are species-specific, only eighteen such protein families were found (Gollery *et al*., 2006). Eight of these families were highly H_2O_2-responsive, but contained only yeast proteins, suggesting that they might be part of a yeast-specific response to H_2O_2 (data not shown). The ten remaining protein families had homologues from at least two kingdoms and were retained for further analysis. For these ten unknown protein families, the conservation of the H_2O_2 induction was restricted to yeast, or to yeast and *A. thaliana*, again demonstrating that the H_2O_2 response is better conserved between yeast and *A. thaliana* than between yeast and *H. sapiens* (Table 5).

The term “protein with unknown function” is used broadly and is mostly based on lack of clear homology with known proteins. A better definition for unknown proteins is “proteins with obscure features” (POFs), which lack defined motifs or protein domains (Gollery *et al*., 2006). To identify POFs, the proteins within the different unknown families were subjected to BLAST homology searches. In doing so, we were able to identify functional domains and could assign putative functions to eight out of ten unknown protein families. One family contained proteins with no functional domains and these are considered to be POFs. A second protein family represented proteins with only predicted membrane function and unknown DUF962 domains.

The conserved H_2O_2 induction of these POFs suggests an important role for them during oxidative stress in yeast or plants. Therefore, these proteins are maybe good candidates to study new aspects of stress signaling and it would be interesting to further analyze the function of these proteins, for example to improve stress tolerance in these species.

Table 5. Conserved H_2O_2 response of unknown protein families, shown as fraction of H_2O_2-induced genes

Family ID	Family description[a]	*S. cerevisiae*	*S. pombe*	*A. thaliana*
976	Unknown protein, putative choline-transporter	1.00	1.00	0.00
1033	Unknown protein, putative glutamate binding, inner membrane localization	N.R.	1.00	0.20
2139	Unknown, contains RING-zinc finger	1.00	1.00	0.00
3312	Unknown protein, predicted membrane function, contains unknown DUF962 domain	1.00	1.00	0.00
3319	Unknown protein, UAS/UBX domain	1.00	1.00	N.R.
4314	Unknown, conserved eukaryotic protein with LisH, CTLH and RING-zinc finger motif	1.00	1.00	0.00
5028	Unknown, pyridoxine 5'-phosphate oxidase-related	1.00	1.00	0.00
5899	Unknown	1.00	1.00	0.00
8617	Unknown, contains ubiquitin, WLM metallopeptidase and PUG domains.	N.R	1.00	1.00
9064	Unknown, contains UbiE/COQ5 Methylase/methyltransferase domains	N.R.	1.00	0.50

[a] Family description is based on protein annotation and blast homology searches. N.R., no representative protein found. DUF, domain of unknown function; UAS, ubiquitin-associated; UBX, ubiquitin regulatory X; LisH, lissencephaly-1 protein homologue; CTLH, c-terminal to LisH; PUG, peptide N-glycanases and other putative nuclear UBA or UBX

CONCLUSIONS

The comparative analysis of H_2O_2-induced gene expression across kingdoms hints at a strongly specialized transcriptional response, besides a small core eukaryotic H_2O_2 response. In addition, this analysis clearly reveals that the inductive transcriptional response to H_2O_2 is highly conserved in yeasts and that this yeast response is more conserved in plants than in animals.

Antioxidant gene expression is only induced in unicellular organisms and not in higher eukaryotes, indicating that some specific responses are only partially conserved. Furthermore, the presented approach was used for gene discovery by focusing on unknown proteins, hereby hypothesizing that genes with a conserved H_2O_2-induced transcription might have an important role during oxidative stress. As more sequence and transcriptome data of other species are expected in the future, sampling within one specific kingdom, phylum, or taxon will lead to new insights into the evolution and conservation of the transcriptional response to oxidative stress.

MATERIALS AND METHODS

Microarray and protein data sets

Expression data of H_2O_2-induced genes were obtained from either websites or from Supplementary data of the corresponding articles: *Synechocystis* (Li *et al*., 2004; available at http://jb.asm.org); the complete data set of *S. cerevisiae* and *S. pombe* from http://www.genome.stanford.edu/yeast_stress/data/rawdata/complete_dataset.txt and from ftp://ftp.sanger.ac.uk/pub/postgenomics/s_pombe/wtaverage.txt, respectively; the complete microarray data set of the human HeLa cell line from http://microarray-pubs.stanford.edu/human_stress/Home.shtml; and a completely processed data set of the microarray analysis in *A. thaliana* (Kim *et al*., 2005; http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2295/TPJ2295sm.htm). A two-fold change cutoff was used to identify genes that were differentially expressed.

The protein data set consisted of sequences from one cyanobacterium (*Synechocystis* sp. strain PCC 6803), two yeasts (*S. cerevisiae* and *S. pombe)*, two mammals (*H. sapiens* and *Mus musculus*) and two plants (*A. thaliana* and rice [*Oryza sativa*]). Sequence information for all *Synechocystis* proteins was obtained from NCBI (http://www.ncbi.nlmn.nih.gov, release NC_0009111). Protein sequences from *S. cerevisiae* and *S. pombe* were retrieved from *Saccharomyces* Genome Database (http://www.yeastgenome.org; release of August 2004) and Gene DataBase (http://www.genedb.org; release of November 2004), respectively. All protein sequences from *H. sapiens* (U25 NCBI 34 assembly) and *M. musculus* (U25 NCBI m33 assembly) were obtained from EnsEMBL (http://www.ensembl.org). Sequence information for *Arabidopsis* (release 5 of January 2004; Wortman *et al*., 2003) and rice (release 2 April 2004; Yuan *et al*., 2003) was provided by The Institute for Genome Research. When multiple protein sequences were available for the same gene locus, the longest was retained.

Construction of Protein Families

Protein families were constructed by applying Tribe-MCL sequence clustering. Tribe-MCL relies on the Markov clustering algorithm using graph-clustering methods and identifies clusters in a protein-protein similarity graph in a process that is sensitive to the density and the strength of the connections (Enright *et al*., 2002). A similarity matrix was generated from an all-against-all comparison using BLAST (Altschul *et al*., 1997) with an E-value threshold of 0.01. Clusters were formed with an inflation factor of 3.0. The original MCL algorithm was obtained from http://micans.org/mcl/ and more information concerning Tribe-MCl is also available at http://www.ebi.ac.uk/research/cgg/services/tribe/.

Significance Estimation using Random Sampling

The significance of the number of stress-responsive gene families conserved between two species was estimated with random sampling. Briefly, for both species the number of genes found in our analysis was randomly selected from all the genes present on the microarrays, the corresponding protein families were identified together with the number of conserved families. Based on 1000 random sampling iterations, the significance of the observed overlap was estimated.

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CHAPTER 3

Identification of genes involved in plant defense against necrotrophic pathogens by using virus-induced gene silencing

ABSTRACT

The response of plants to pathogen infection involves an oxidative burst caused by enhanced production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2). The oxidative burst is considered to facilitate necrotrophic infection, but recent reports also suggest a positive role for ROS in plant defense against necrotrophic pathogens. We took advantage of a cDNA-AFLP analysis that was performed to discover H_2O_2-induced gene transcripts in *Nicotiana tabacum* (tobacco) (Vandenabeele *et al*., 2003). Approximately 180 H_2O_2-induced tobacco genes were screened for a possible role during plant defense against two necrotrophic pathogens, *Botrytis cinerea* and *Sclerotinia sclerotiorum*, using virus-induced gene silencing (VIGS) in *Nicotiana benthamiana*. A selection of 25 genes was further tested using VIGS in *Lycopersicon esculentum* VF36 (tomato). VIGS of four genes, encoding a mitogen-activated protein (MAP) kinase kinase kinase (NPK1), a MAP kinase kinase (NQK1), a heat shock protein (HSP) and a putative esterase / lipase protein, resulted in increased sensitivity to *Botrytis* and/or *Sclerotinia*, suggesting a role for these genes in plant defense responses to necrotrophic pathogens. VIGS-inoculated plants were also analyzed for altered growth and development. Silencing of four genes (BYPASS, DNA-directed RNA polymerase, threonyl tRNA synthase, and 26S proteasome regulatory subunit) resulted in phenotypic aberrations, including growth retardation and stunted growth, suggesting that these genes have functions that are important for normal development of plants.

INTRODUCTION

Necrotrophic pathogens

Necrotrophic pathogens, such as *Botrytis cinerea* (*Botrytis*) and *Sclerotinia sclerotiorum* (*Sclerotinia*) kill host plants and decompose dead plant tissues for their own consumption (van Kan, 2006). *Sclerotinia* (known as white mould) and *Botrytis* (known as grey mould) have a broad host range and are difficult to control, making them important pathogens for agriculture and flower cultivation (Bolton *et al*., 2006; Hegedus and Rimmer, 2005; Williamson *et al*., 2007). One of the major problems for efficient disease control is the variety of modes of attack and inoculum sources. Infection of the host can occur via seed transmission (seed-born infection) and during development, which causes enormous losses prior to harvest (Williamson *et al*., 2007). In addition, crop losses also occur after harvesting and transport of apparently uninfected plants to distant markets because necrotrophic pathogens can remain quiescent under unfavorable conditions for long periods. Another limitation of current disease management is the high costs of bringing new fungicides (chemical control agents, CCAs) or BCAs (biological control agents) to the market (Leroux, 2004). Moreover, pathogens can evolve multi-drug resistance mechanisms that lead to increased insensitivity to CCAs and BCAs.

Because of the worldwide impact of *Botrytis* disease, it has become the most extensively studied necrotrophic fungal pathogen and it is now regarded a model for necrotrophic pathogens with a broad host range (van Kan, 2006). Molecular tools to study *Botrytis* are available, hence many virulence genes have been identified and also the infection process itself is under investigation (Baldwin *et al*., 2006). It is now evident that ROS play an important role during the infection process of *Botrytis* (van Kan, 2006; Williamson *et al*., 2007). The tip of the penetration peg, an infection structure on the host surface that breaches the cuticle, generates H_2O_2 which might assist in its penetration by providing a substrate for oxidases that modify and weaken the cuticle (Tenberge, 2004). *Botrytis* infection also results in massive accumulation of ROS at the plant plasma membrane and in the extracellular sheath covering the surface of fungal hyphae, thereby triggering an oxidative burst in and around the infected tissue, as well as in uninfected tissues (Schouten *et al*., 2002; Tenberge 2004). Such an oxidative burst occurs in many plant-pathogen interactions during a hypersensitive response and confers resistance to biotrophic pathogens. In contrast, the oxidative burst generated by necrotrophs is considered to assist in primary lesion formation and plant cell death, thereby promoting disease progress (Lyon, 2004). Recent evidence suggests a positive role for ROS in defense against *Botrytis* (Asselbergh *et al*., 2007; Malolepsza and Urbanek, 2002; Unger *et al*., 2005). It was shown that a rapid H_2O_2 accumulation assists in the resistance of the *sitiens* mutant to *Botrytis* by altering the expression of defense genes (Asselbergh

et al., 2007). In addition to promoting cell death, H_2O_2 may thus also act as a signaling molecule that activates signaling pathways during the host's defense response against necrotrophic pathogens.

Virus-induced gene silencing: An efficient tool to study the defense response of (higher) plants

The VIGS technology exploits the plant anti-viral response to silence endogenous genes (Baulcombe 1998). Upon infection, viral replication occurs through the formation of double stranded RNA, which is recognized by the host and subsequently targeted for degradation into small RNA molecules via the RNA silencing pathway. For VIGS, a short sequence of a target plant gene is cloned into a modified virus genome, which is then mechanically introduced in the plant to allow the virus to replicate. Degradation of viral RNA by the host's silencing machinery would then also result in small RNA molecules of the incorporated plant gene, which are called small interfering RNA molecules, and these direct the silencing complex to the endogenous plant mRNA to induce mRNA degradation.

VIGS is a powerful technology to study gene function in plants with many advantages over classical functional genomics approaches (Burch-Smith *et al*., 2004). Because VIGS circumvents stable plant transformation, it is extremely amenable for fast, high-throughput screens (Fitzmaurice *et al*., 2002; Lu *et al*., 2003). In addition, VIGS can overcome functional redundancy by silencing of homologous genes and can be used to study homologues genes in related species. However, one should also be aware of some limitations associated with VIGS. It does not always lead to complete and uniform gene suppression, which can complicate data interpretation or even mask the results. In addition, it cannot be ruled out that VIGS leads to suppression of non-target genes.

At least 18 different viruses have been modified to serve as silencing vectors (Table 1). Most (~70%) silencing vector are based on RNA viruses and these include viruses from the genus Tobra-, Tobamo-, Hordeivirus, Tobus-, Carla-, Potex-, Como-, Chera- or Bromovirus. RNA viruses replicate in the cytoplasm using their own RNA polymerase and host structures, including cytoplasmic membranes, ribosomes and proteins. DNA viruses used for VIGS include viruses of the genus Begomovirus (family Geminiviridae) which replicate in the nucleus using host DNA replication machinery. VIGS systems based on satellite viruses, such as satellite tobacco mosaic virus and DNAβ satellite virus, have also been developed (Carillo-Tripp *et al*., 2006; Gosselé *et al*., 2002; Tao *et al*., 2004). Satellite viruses have a small genome that usually encodes their own coat protein, but they rely on a second virus for replication.

One of the most popular and best performing VIGS vectors is based on the Tobacco Rattle Virus (TRV; Liu *et al*., 2002a; Ratcliff *et al*., 2001). TRV overcomes limitations of other VIGS vectors

based on Tobacco Mosaic Virus, Potato Virus X and Geminiviridae because it only shows mild infection symptoms and can spread throughout the plant, including meristimatic tissues. In addition, TRV hast a broad host range which includes several members from the *Solanaceae* family (tobacco, potato and tomato) as well as *Arabidopsis thaliana* and different *Ranunculaceae* (Cai *et al*., 2006; Hileman *et al*., 2005; Liu *et al*., 2002a; Ratcliff *et al*., 2001; Ryu *et al*., 2004; Wege *et al*., 2007).

Solanaceae, including *N. benthamiana*, and tomato (*L. esculentum*) are highly susceptible to virus infection and most VIGS vectors work in these species. *N. benthamiana* is extremely popular because it can be easily infiltrated with the VIGS constructs and VIGS phenotypes are extremely pronounced (Lu *et al*., 2003; Robertson *et al*., 2004). In addition, it can serve as a model plant for *Solanaceae* crops, such as tomato and potato. The number of plant species that can be subjected to VIGS is increasing thanks to the recent development of host-specific VIGS vectors, making it possible to study gene function in economically important plant species. Vectors are now available for manioc (African cassava mosaic virus), legumes (Pea early browning virus), rice (Brome mosaic virus), soybean (Cucumber mosaic virus and Bean pod mottle virus), orchids (Cymbidium mosaic virus), barley (Barley stripe mosaic virus) and wheat (Barley stripe mosaic virus) (Table 1).

VIGS vectors can be inoculated in plants via different procedures (Table 2). The chosen method is dependent on the virus system and plant host. DNA viruses, such as Geminiviridae, are usually been brought into the plant using biolistics or bombardment with DNA coated microprojectiles (Kjemtrup *et al*., 1998; Peele *et al*., 2001; Turnage *et al*., 2002; Fofana *et al*., 2004; Carillo-Tripp *et al*., 2006). Originally, VIGS vectors based on RNA viruses required *in vitro* transcription of infectious RNA from linearized plasmids, which are then mechanically introduced into plant cells by rubbing with carborundum (Kumagai *et al*., 1995). However, the most used and probably the most potent technique is direct infiltration with *Agrobacterium*, either with a needle, toothpick, syringe (without needle), or by using vacuum infiltration, agrodrench or spraying. Alternatively, plants can be sap-inoculated with extracts from agro-infiltrated leaves which reduces secondary effects, such as necrosis and plant stunting (Brigneti *et al*., 2004). Agro-inoculation has advantages over *in vitro* transcription because the virus vector cDNA does not have to be isolated, digested, or transcribed. Moreover, *Agrobacterium* inoculation allows T-DNA transformation of plant cells at the site of inoculation, which then can promote systemical spread of the infection throughout the whole plant. It can also be used to promote gene silencing in other tissues than leaves, such as roots and fruits (Fu *et al*., 2005; Ryu *et al*., 2004). Finally, agro-inoculation is particularly useful in high-throughput VIGS applications when, for example, cDNA libraries or EST collections are to be tested.

Table 1. Characteristics of viruses used for silencing vectors.

Virus	Genus[1]	Host[2]	Reference
RNA viruses[1]			
Tobacco rattle virus (TRV)	Tobra	*N. benthamiana*	Ratcliff *et al*., 2001
		L. esculentum	Liu *et al*., 2002a
		C. annuum	Chung *et al*., 2004
		N. tabacum	Ryu *et al*., 2004
		S. tuberosum	Ryu *et al*., 2004
		P. hybrida	Ryu *et al*., 2004
		S. bulbocastanum	Brigneti *et al*., 2004
		S. okadae	Brigneti *et al*., 2004
		S. nigrum	Brigneti *et al*., 2004
		P. somniferum	Hileman *et al*., 2005
		A. thaliana	Lu *et al*., 2003
		A. thaliana	Cai *et al*., 2006
		E. californica	Wege *et al*., 2007
		A. vulgaris	Gould and Kramer, 2007
		N. benthamiana	Valentine *et al*., 2004
		L. esculentum	Valentine *et al*., 2004
Pea early browning virus (PEBV)	Tobra	*P. sativum*	Constantin *et al*., 2004
Tobacco mosaic virus (TMV)	Tobamo	*N. benthamiana*	Kumagai *et al*., 1995
Satellite tobacco mosaic virus (STMV)	Tobamo	*N. tabacum*	Gosselé *et al*., 2002
Barley stripe mosaic virus (BSMV)	Hordei	*H. vulgare*	Holzberg *et al*., 2002
		T. aestivum	Scofield *et al*., 2005
Tomato bushy stunt virus (TSBV)	Tombus	*N. benthamiana*	Hou *et al*., 2003
Poplar mosaic virus (PopMV)	Carla	*N. benthamiana*	Naylor *et al*., 2005
Potato Virus X (PVX)	Potex	*S. tuberosum*	Faivre-Rampant *et al*., 2004
		S. bulbocastanum	Faivre-Rampant *et al*., 2004
		A. thaliana	Dalmay *et al*., 2000
		N. benthamiana	Ruiz *et al*., 1998
		N. tabacum	Angell *et al*., 1999
		L. esculentum	Angell *et al*., 1999
Cymbidium mosaic virus (CymMV)	Potex	*Phalaenopsis*	Lu *et al*., 2007
Bean pod mottle virus (BPMV)	Como	*G. max*	Zhang *et al*., 2006
Apple latent spherical virus (ALSV)	Chera	*N. benthamiana*	Yaegashi *et al*., 2007
Brome mosaic virus (BMV)	Bromo	*O. sativa*	Ding *et al*., 2006
Cucumber mosaic virus (CMV)	Bromo	*G. max*	Nagamatsu *et al*., 2007
DNA viruses[1]			
African cassava mosaic virus (ACMV)	Begomo	*M. esculenta*	Fofana *et al*., 2004
		N. benthamiana	Fofana *et al*., 2004
Pepper huasteco yellow vein virus (PHYVV)	Begomo	*C. annuum*	Carrillo-Tripp *et al*., 2006
		N. tabacum	Carrillo-Tripp *et al*., 2006
		L. esculentum	Carrillo-Tripp *et al*., 2006
DNAβ satellite virus associated with Tomato yellow leaf curl China virus (TYLCCNV)	Begomo	*L. esculentum*	Cai *et al*., 2007
Cabbage leaf curl virus (CaLCuV)	Begomo	*A. thaliana*	Turnage *et al*., 2002
Tomato golden mosaic virus (TGMV)	Begomo	*N. benthamiana*	Peele *et al*., 2001

[1] Type (RNA or DNA) and Genus were obtained from http://www.ncbi.nlm.nih.gov/Taxonomy/.

[2] Hosts as described in the references.

Table 2. Techniques for VIGS inoculation.

Inoculation Method	Target Tissue	Reference
Biolistics / Particle bombardement	Leaves	Kjemtrup *et al.*, 1998
In vitro transcripts inoculation	Leaves	Kumagai *et al*., 1995
Agrobacterium-inoculation with toothpick	Leaves	Faivre-Rampant *et al*., 2004
Agrobacterium-infiltration with needle	Leaves	Tao *et al*., 2004
Agrobacterium-infiltration with syringe	Leaves; Fruit	Ratcliff *et al*., 2001; Fu *et al*., 2005
Agrobacterium Spraying	Leaves	Liu *et al*., 2002
Agrodrench	Roots and Leaves	Ryu *et al*., 2004
Vacuum infiltration	Leaves	Hileman *et al*., 2005
Sap inoculation	Leaves	Brigneti *et al*., 2004

In recent years, VIGS has been proven to be an excellent tool for studying gene function (reverse genetics) and identifying new genes associated with a particular phenotype (forward genetics) (Benedito *et al*., 2004; Burch-Smith *et al*., 2004). VIGS has been used to silence genes involved in root, flower, leaf and overall plant development, as well as genes involved in hormone signaling, metabolite synthesis, tolerance to abiotic stress and cell death (Ahn *et al*., 2004; Ahn *et al*., 2006; Burger *et al*., 2003; Chen *et al*., 2004; Constantin *et al*., 2004; Darnet and Rahier, 2004; Fofana *et al*., 2004; Fu *et al*., 2006; Gosselé *et al*., 2002; Kim *et al*., 2006; Lee *et al*., 2003; Park *et al*., 2005; Senthil-Kumar *et al*., 2007; Valentine *et al*., 2004). However, the most reported application of VIGS is the study of plant defense responses against pathogens (Table 3). VIGS has particularly been useful to find genes that enhance or attenuate the hypersensitive response during *R*-gene mediated resistance against avirulent pathogens (Brigneti *et al*., 2004; Ekengren *et al*., 2003; Gabriëls *et al*., 2006; Liu *et al*., 2004; Sacco *et al*., 2007).

The aim of this study was to investigate the role of H_2O_2 during plant defense against *Botrytis* and *Sclerotinia*. Previously, a cDNA-AFLP analysis was performed in the lab for the identification of H_2O_2-induced genes (Vandenabeele *et al*., 2003) and this was now combined with a VIGS approach to screen for genes that alter the defense response of plants to necrotrophic pathogens. A cartoon of the followed strategy is depicted in Figure 1. H_2O_2-induced genes were cloned into the VIGS constructs based on the TRV virus and inoculated in plants to induce gene silencing. Infiltrated plants were scored for developmental aberrations and assayed for altered resistance against necrotrophic pathogens. VIGS constructs were first evaluated by high-throughput screening in *N. benthamiana* and a subset of them was further tested in an economically relevant crop species, *L. esculentum* VF36 (tomato).

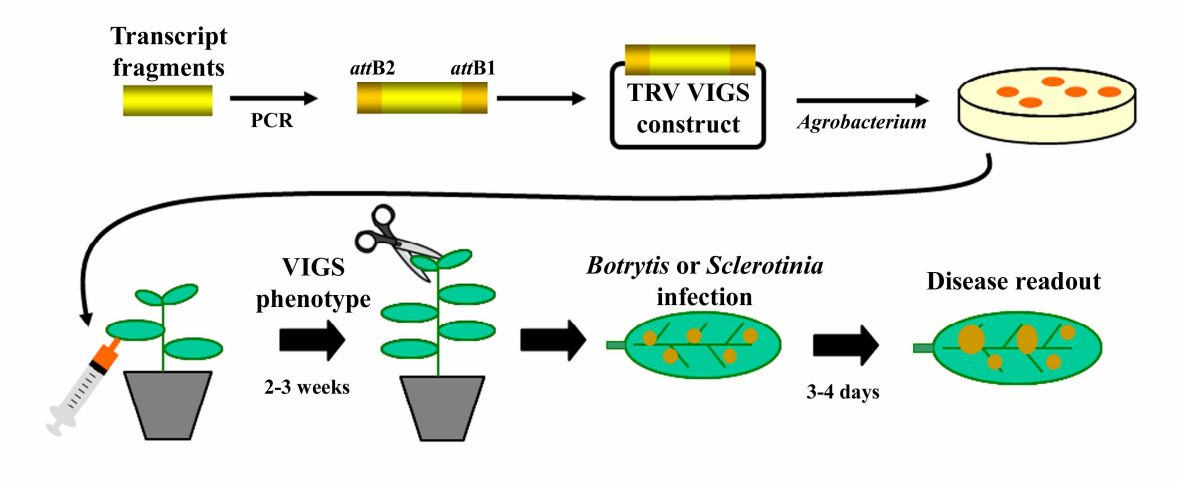

Figure 1

Strategy of this study. Transcript fragment were cloned into the tobacco rattle virus (TRV) vector using the Gateway® technology. The resulting constructs were used for virus-induced gene silencing (VIGS) in plants via inoculation with *Agrobacterium*. The phenotype of plants that were infiltrated with the TRV VIGS constructs was scored 2-3 weeks after infiltration. VIGS infiltrations were followed by necrotrophic pathogen infections (*Botrytis* and *Sclerotinia*) on detached leaves to screen for genes that affect the defense response of plants.

Table 3. Use of VIGS to study gene function during plant defense

Target Gene	Function	VIGS Phenotypes	References
SIGNALING			
ACIK1	Avr9/Cf-9 induced kinase 1	Decreased *Cf*-9 mediated resistance to Cladosporium fulvum (biotrophic fungus)	Rowland *et al*., 2005
APR134	Calmodulin-related protein	Compromised *Pto*-mediated resistance to *P. syringae*	Chiasson *et al*., 2005
CDPK2	Calcium-dependent protein kinase 2	Delayed *Cf*-4 and *Cf*-9 induced HR and wilting	Romeis *et al*., 2001
CTR1	MAPKKK (Constitutive Triple Response 1)	Constitutive ethylene response, enhanced N-mediated HR to TMV	Liu *et al*., 2002b; Liu *et al*., 2004
COI1	Jasmonic acid signaling	Compromised *R*-gene mediated resistance to various pathogens	Ekengren *et al*., 2003; Liu *et al*., 2004
MAPKKKalfa	MEKK A2 class MAPKKK	Loss of *Pto*-induced cell death and resistance against *P. syringae*; decreased disease-associated cell death	del Pozo *et al*., 2004
MEK1/NQK1	MAPKK protein involved in cytokinesis	Compromised resistance to TMV (*N* gene) and *P. syringae* (*Pto* gene)	Liu *et al*., 2004; Ekengren *et al*., 2003
MEK2	MAPKK family protein	Compromised *Pto*-induced resistance to *P. syringae*	Ekengren *et al*., 2003
WIPK	Wound-Induced Protein Kinase, MAPK family	Reduced resistance to *P. syringae* (*Pto*)and *P. cichorii* (bacterial pathogen), no effect on *INF1*-induced HR	Ekengren *et al*., 2003; Sharma *et al*., 2003
MKK1	MAPKK	No *INF1*-induced HR, compromised non-host resistance to *P. cichorii*	Takahashi *et al*., 2007
MPK1/2/3	MAPK proteins	Involved in systemin-related defense response and protection against *M. sexta* herbivorous insects	Kandoth *et al*., 2007
SABP3	Salicylic acid-binding protein 3	Attenuated *Pto*-induced HR	Slaymaker *et al*., 2002
SIPK	Stress-induced protein kinase	Reduced resistance to *P. cichorii*, no effect on *INF1*-induced HR	Sharma *et al*., 2003
NPK1/MEKK1	MAPKKK protein involved in cytokinesis	Interferes with disease resistance genes *N*, *Bs2*, *Rx*, but not *Pto* and *Cf*4; Increased susceptibility to TMV	Jin *et al*., 2002
NTF6/NRK1	MAPK protein involved in cytokinesis	Compromised resistance to TMV (*N*) and *P. syringae* (*Pto*)	Liu *et al*., 2004; Ekengren *et al*., 2003
PP2A	Protein Phosphatase 2A	Increased resistance to *P. syringae* (*Pto*) and *C. fulvum* (*Cf*-9) through constitutive PR gene induction, enhanced cell death and HR	He *et al*., 2004
RanGAP2	Ran GTPase-activating enzyme	Compromised *Rx*-mediated resistance to PVX	Sacco *et al*., 2007
TRANSCRIPTION			
CAF1	CCR4-associated factor 1	Growth retardation and enhanced susceptibility to *X. axonopodis*	Sarowar *et al*., 2007
CD1	Ethylene responsive-element binding factor	Required for non-host resistance of *N. benthamiana* to *P. cichorii*	Nasir *et al*., 2005
EIL	Transcription factor in ethylene signaling	Compromised resistance to *P. hyoscyami*	Borras-Hidalgo *et al*., 2006
WRKY1/2/3	WRKY family Transcription factor	Compromised *N*-mediated resistance to TMV	Liu *et al*., 2004
MYB1	MYB family Transcription factor	Compromised *N*-mediated resistance to TMV	Liu *et al*., 2004
TGA.2.2	Transcription Factor	Compromised resistance to *P. syringae* (*Pto*)	Ekengren *et al*., 2003
TGA1a	Transcription Factor	Compromised resistance to *P. syringae* (*Pto*)	Ekengren *et al*., 2003
NPR1/NIM1	Transcription factor, SA-mediated defense	Compromised *R*-gene function and resistance to TMV (*N*) and *P. syringae* (*Pto*)	Ekengren *et al*., 2003; Liu *et al*., 2002b
PPS3	Putative GATA-type transcription factor	Delayed StMEK1(DD)- or hyphal wall elicitor-induced HR-like cell death	Katou *et al*., 2005
PROTEIN DEGRADATION			
Cathepsin B	Papain cysteine protease	Compromised PCD and disease resistance against non-host bacterial pathogens, suppressed *Avr3a*/*R3a* mediated HR and reduced induction of *Hsr203* following *E. amylovora* challenge	Gilroy *et al*., 2007
MCA1	Proprotein processing, apoptosis	Increased susceptibility *to C. destructivum*, no effect on HR and *P. syringae* infection	Hao *et al*., 2007

Target Gene	Function	VIGS Phenotypes	References
REDOX CONTROL			
CITRX	Thioredoxin	Accelerated HR, ROS accumuation, induction of defense genes, increased *Cf*-9 mediated resistance to *C. fulvum*	Rivas *et al*., 2004
GSHS	Glutathione synthetase	Compromised resistance to *P. hyoscyami*	Borras-Hidalfo *et al*., 2006
GSTU1	Glutathione-S-transferase	Increased susceptibility to *C. orbiculare*	Dean *et al*., 2005
RbohA/B	ROS biosynthesis	Compromised HR and Inf1-induced resistance to *P. infestans*	Yoshioka *et al*., 2003
DEFENSE			
GLP	Germin-like Protein	Enhanced susceptibility to herbivorous pathogens	Lou *et al*., 2006
Lr21	Resistance gene of the NB-LRR family	Loss of *Lr21*-mediated resistance to *P. triticina*	Scofield *et al*., 2005
RAR1	Necessary for Resistance gene function	Required for multiple *R*-gene resistance pathways and resistance to various pathogens	Liu *et al*., 2002b; Hein *et al*., 2005; Ekengren *et al*., 2003; Scofield *et al*., 2005; de la Fuente van Bentem *et al*., 2005
Rb	Resistance gene	Attenuated *R*-gene-mediated resistance to *P. infestans*	Brigneti *et al*., 2004
R1	Resistance gene	Attenuated *R*-gene-mediated resistance to TMV and *P. infestans*	Brigneti *et al*., 2004
NRC1/ART	NB-LRR protein required for HR-associated Cell death 1	Required for the HR induced by *Cf*-4, *Cf*-9, *LeEix*, *Pto*, *Rx* and *Mi* and *Cf*-4 mediated resistance to *C. fulvum*	Gabriëls *et al*., 2007
Mi	Nematode resistance	Loss of *Mi*-induced resistance to nematodes	Valentine *et al*., 2004
NRG1	N Requirement Gene 1, Resistance gene of CC-NB-LRR family	Loss of *N*-mediated resistance to TMV	Peart *et al*., 2005
Rx	Resistance gene	Attenuated *R*-gene-mediated resistance to PVX	Brigneti *et al*., 2004
OTHER CATEGORIES			
HSP70	Heat Shock Protein 70, molecular chaperone	Stunted, no *INF1*-induced HR, compromised non-host resistance to P. cichorii	Kanzaki *et al*., 2003
HSP90	Heat Shock Protein 90, molecular chaperone	Required for multiple R-gene resistance pathways and non-host resistance to various pathogens	Lu *et al*., 2003; Hein *et al*., 2005; de la Fuente van Bentem *et al*., 2005; Kanzaki *et al*., 2003; Scofield *et al*., 2005; Bhattarai *et al*., 2007
33k subunit of PSII	Chloroplast rotein involved in photosynthesis	Higher sensitivity to TMV, AMV and PVX by inhibition of photosystem II, independent of the *N*-gene	Abbink *et al*., 2002
AAA-ATPase	ATPase associated with various activities	Higher resistance to TMV, AMV and PVX, independent of the *N*-gene	Abbink *et al*., 2002
ACO1	ACC oxidase, involved in ethylene biosynthesis	Higher sensitivity to *C. orbiculare* by reductions in defense genes, accelerated switch to necrotic phase	Shan *et al*., 2006
Aconitase	Catalyses the conversion of citrate to isocitrate/ RNA binding protein	Decreased *Pto*-mediated resistance to *P. syringae* and disease associated cell death	Moeder *et al*., 2007
EDS1	Lipase	Required for *N*-mediated resistance to TMV, not for compatibel interactions	Peart *et al*., 2002a; Liu *et al*., 2002b; El Oirdi and Bouarab, 2007
FAD1	Fatty acid biosynthesis	Compromised resistance to TMV and *Bax*-induced PCD	Kim *et al*., 2007
SGT1	Involved in R-mediated disease resistance	Required for multiple *R*-gene resistance pathways and non-host resistance to various pathogens	Hein *et al*., 2005; Leister *et al*., 2005; El Oirdi and Bouarab, 2007; Scofield *et al*., 2005; de la Fuente van Bentem *et al*., 2005; Bhattarai *et al*., 2007; Peart *et al*., 2002b
VarP	Protein of the glycine decarboxylase complex (GDC)	Increased disease-symptoms and *P. syringae* (*Pto*) growth in resistant and susceptible tomato	Chandok *et al*., 2004

Table 3 continued.

RESULTS

Selection and cloning of gene fragments

In total, 234 H_2O_2-induced genes were selected for functional analysis during defense against necrotrophic pathogens. These genes were selected from a cDNA-AFLP analysis on high light treated catalase-deficient plants which was performed to identify H_2O_2-regulated genes during cell death in tobacco (*Nicotiana tabacum*) (Vandenabeele *et al*., 2003). In order to make a well-considered choice, genes were picked based on their temporal expression profile (i.e. early and strong induced genes). The sequences were analyzed using BLAST searches against public sequence databases to find homology with longer tobacco ESTs or with the complete cDNAs, which were subsequently used to search for homologous genes in *Arabidopsis*, for which more molecular data is available compared to tobacco or tomato. In a parallel approach, genes were included from a literature search that aimed at identifying genes whose products are induced by abscisic acid (ABA) during various stresses (Curvers, 2004). Information of the selected genes can be found in Supplementary Table S4.

Transcript fragments of the selected genes were PCR amplified and inserted into a gateway entry vector (pDONR207) to allow high-throughput cloning in the TRV RNA2-derived vector (pTV00GW2), a gateway-compatible version of the original pTV00 vector (Ratcliff *et al*., 2001). Gene fragments for 200 of the 234 selected fragments were successfully cloned. pTV00GW2 constructs were transformed into *Agrobacterium* for VIGS by agro-inoculation in plants.

Identification of genes that are necessary for normal plant growth and development

To optimize the VIGS procedure to our experimental conditions, we performed VIGS of a gene encoding a phyotene desaturase (PDS), which is essential for carotenoid biosynthesis. Silencing of PDS causes photobleaching and results in white mosaic patterns that are clearly visible throughout the whole plant (Kumagai *et al*., 1995). The same VIGS procedure as described by Racliff and coworkers (2001) was followed (see Materials and Methods for more details). VIGS using these conditions resulted in extensive photobleaching in approximately nine of ten TRV-PDS infiltrated plants, while, except from a initial growth retardation, no relevant effect on normal plant development was observed after infiltration with the empty TRV vector (Figure 2*A*). The first bleaching symptoms in PDS silenced plants were observed 10-14 days after infiltration, and the symptoms spread throughout new developing tissues in the complete plant. No bleaching was however observed in older tissues. Because of the transparency of the phenotype, silencing of PDS was used as a positive control for all the following VIGS experiments.

After optimization, 180 of the 200 VIGS constructs were successfully screened by direct agro-infiltration in *N. benthamiana* (three plants per construct). All plants were phenotypically scored after the onset of bleaching in PDS-silenced plants. We found that VIGS with four constructs, containing sequences for BYPASS, a DNA-directed RNA polymerase (DRP), a threonyl-tRNA synthetase (ThrRS) and a proteasome 26S regulatory subunit (26S RSU), resulted in aberrant effects on normal growth and development in all infiltrated plants (see Figure 2*B* for phenotypes and Table 4 for details on the genes).

Tabel 4. Genes for which VIGS induced developmental defects

ID	Annotation	*Arabidopsis*
BC4-M44-046	BYPASS protein	AT1G01550
BC4-M42-042	DNA-directed RNA polymerase (DRP)	AT3G59600
BC3-M24-052	Threonyl-tRNA synthetase (ThrRS)	AT5G26830
BT1-M21-048	26S proteasome regulatory subunit (26S RSU)	AT2G32730

VIGS of BYPASS, DRP, ThrRS and 26S RSU caused a severe growth arrest and resulted in stunted plants that were at least two times smaller than empty vector inoculated plants. Silencing of BYPASS and DRP inhibited shoot development (almost no development of new leaves and inflorescence), but older leaves were unaffected. Silencing of the ThrRS completely abolished the development of stem and leaf petioles which resulted in a lettuce-like phenotype. A strong inhibition of stem growth was also observed after VIGS of the 26S RSU, which resulted in miniature plants, but leaf growth and development seemed unaffected. As TRV-mediated VIGS can be performed in a wide range of *Solanaceous* plant species and because heterologous gene sequences can be used to silence their respective orthologs in related plant species (Senthil-Kumar *et al.,* 2007), we assessed the possibility of using tobacco sequences to silence endogenous tomato genes by using a TRV construct carrying the tobacco PDS sequence. The PDS sequence was cloned in a TRV VIGS vector (pTRVRNA2-GW) which was improved for VIGS in tomato (Liu *et al*., 2002a). At least eight (on a total of ten) infiltrated plants showed bleaching and we therefore concluded that the silencing was efficient in tomato (data not shown). Also the sequence of BYPASS, DRP, ThrRS and 26S RSU were cloned in pTRVRNA2-GW for VIGS in tomato. Similar as observed in *N. benthamiana*, VIGS of BYPASS, DRP, ThrRS and 26S RSU in tomato resulted in abnormal effects on growth and development (Figure 2). For DRP and the ThrRS, the phenotype was observed in all ten infiltrated plants, while for BYPASS and 26S RSU, it was observed in approximately half of the infiltrated plants (data not shown). For BYPASS, we did not observe a complete growth inhibition, but plants were stunted and showed a curly leaf phenotype. In conclusion, our results showed that tobacco cDNA-AFLP fragments can be used to silence the homologues tomato genes and that BYPASS, DRP, ThrRS and 26S RSU are necessary for plant growth and development.

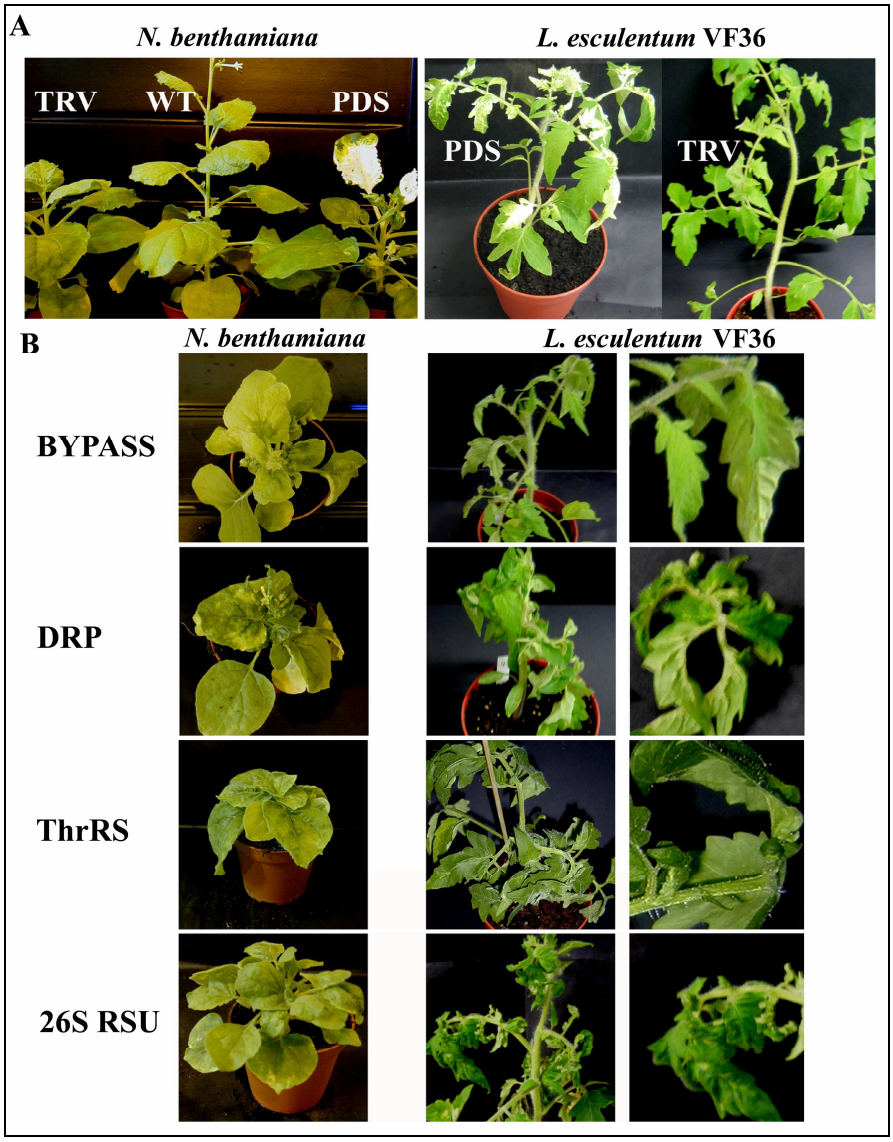

Figure 2

Virus-induced gene silencing (VIGS) phenotypes. **A** Phenotype of wild type (WT) plants, plants infiltrated with the empty TRV construct (TRV), and with the TRV silencing vector for phytoene desaturase (PDS). **B** Phenotype of plants infiltrated with TRV silencing vectors for BYPASS, DNA-dependent RNA polymerase (DRP), threonyl tRNA synthetase (ThrRS) and a 26S proteasome subunit (26S RSU). Photographs were taken 2-3 weeks after VIGS infection. One representative plant per gene is shown.

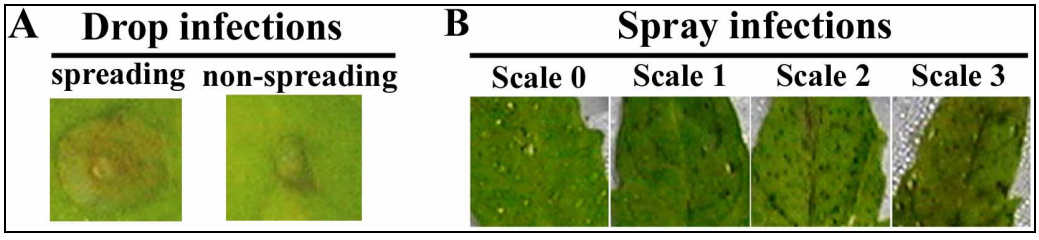

Figure 3

Symptoms of necrotrophic infections. **A** Scoring of a spreading and non-spreading lesion as observed during drop infections in *N. benthamiana*. **B** Scoring of spray infections with *Botrytis* (in tomato): scale 0 = no lesions; scale 1 = only some small spots; scale 2 = 5-40% affected leaf; scale 3 = more than 40% affected leaf.

High-throughput VIGS screening for genes that altered the defense response of *N. benthamiana*

The goal of this study was to use VIGS to screen for genes that are involved in the defense response of plants against necrotrophic pathogens. VIGS-infiltrated plants (three plants / construct) were used for pathogen infections with *Botrytis* and/or *Sclerotinia* assays (see Materials and Methods for more details). Per plant, the leaf at position nine or ten (one being the oldest leaf) was detached three weeks after VIGS-infiltration, which was coincident with clear observation of bleaching throughout PDS-silenced plants. Detached leaves (3-5 / construct) were inoculated with infection suspensions by pipetting eight-ten droplets on the surface of the leaf. Disease symptoms were scored 4-5 days after pathogen infection by classifying each inoculation droplet as a spreading or non-spreading lesion, corresponding to a sensitive or resistant reaction of the plant, respectively (Figure 3*A*). Alternatively, we used spray inoculations of *Botrytis* infections to allow a better coverage of the leaf (Asselbergh *et al*., 2007). Spray infections were digitally photographed and the images were scored via APS Assess which allows quantifying disease symptoms on leaves as percentage of total leaf area (Lamari, APS, St. Paul, Minn.).

Disease symptoms on each infected leaf were compared with the average of the corresponding controls. In order to make a well-considered selection for confirmation in tomato, results for one construct were considered as different from controls when the disease symptoms on at least two leaves (on a total of three leaves) were higher or lower than the average of the corresponding controls (Figure 4; Table 5). Based on this criterion, 25 constructs were retained. VIGS-infiltration with 11 constructs resulted in increased sensitivity towards *Botrytis*, while VIGS with two other constructs increased sensitivity towards *Sclerotinia*. VIGS with 12 constructs resulted in increased resistance against *Botrytis*. Due to the low number of infected plants per construct, no statistical validation of the results was possible.

The corresponding genes were classified according to their putative functions based on gene ontology (GO) functional terms (Figure 5). Abundant GO classes are plant defense, signal transduction and protein metabolism. One third of the sequences could not be annotated and were therefore classified as proteins with unknown function.

Tabel 5. 25 genes for which VIGS altered the defense response of *N. benthamiana*

ID	Annotation	AGI
***Botrytis* drop infection[1]**		
NRK	MAPK	AT1G07880
NQK	MAPKK	AT5G56580
***Sclerotinia* drop infection[1]**		
BT4-M23-026	Unknown, contains domain Hs1pro-1	AT2G40000
BT1-M34-037	Unknown	No significant hit
***Botrytis* spray infection[2]**		
Sensitive		
BC1-M41-018	Ubiquitin-conjugating enzyme	AT3G17000
BC2-M42-018	Putative In2-1 protein	AT5G02790
BC1-M43-024	low similarity to ERF/AP2 transcription factor	AT5G50080
BC4-M34-045	Unknown, contains esterase/lipase/thioesterase domain	AT3G27320
BT1-M21-048	26S proteasome regulatory subunit	AT2G32730
BT1-M22-007	Cytochrome b6 apoprotein	ATCG00720
BT2-M41-008	Small HSP class CIII	AT1G54050
BT3-M22-004	Small HSP class CI	AT1G53540
BT4-M33-006	GRAM domain-containing protein /ABA-responsive protein-related	AT2G22475
Resistant		
BC4-M14-069	Protein kinase	AT5G02800
BT1-M21-024	Protein kinase	AT2G17220
BC2-M42-022	Unknown	No significant hit
BC3-M32-022	Unknown	No significant hit
BC2-M14-026	Iron hydrogenase	AT4G16440
BC3-M13-022	Lipase class 3 family protein / calmodulin-binding heat-shock protein	AT3G49050
BC2-M22-020	Unknown	No significant hit
BC3-M33-106	Unknown	No significant hit
BT1-M21-020	Permease-related	AT3G26670
BC2-M13-038	Unknown	No significant hit
BC2-M22-028	Cystathionine gamma-synthase isoform 1	AT3G01120

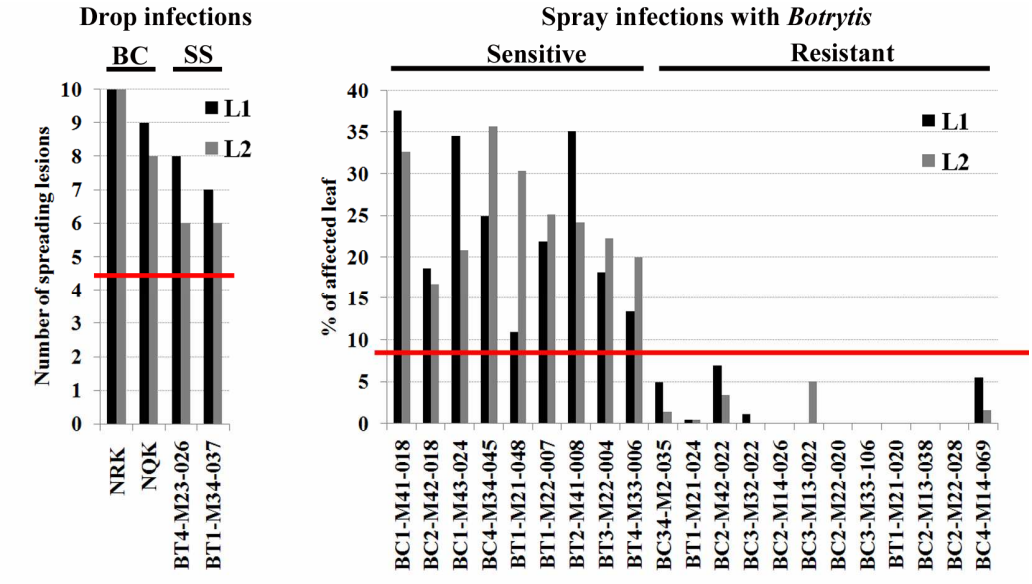

Figure 4

Infection results for 25 genes that altered the defense response of *N. benthamiana*. Genes were silenced using VIGS and detached leaves were used for pathogen infections with *Botrytis cinerea* (BC) or *Sclerotinia sclerotiorum* (SS). Results for the infections are shown as number of spreading lesions for drop infections, or as percentage affected leaf surface for spray infections. Per gene, results are shown for two infected leaves (on a total of three leaves). The red line represents the average disease symptoms on control leaves. L1, leaf one; L2, leaf two.

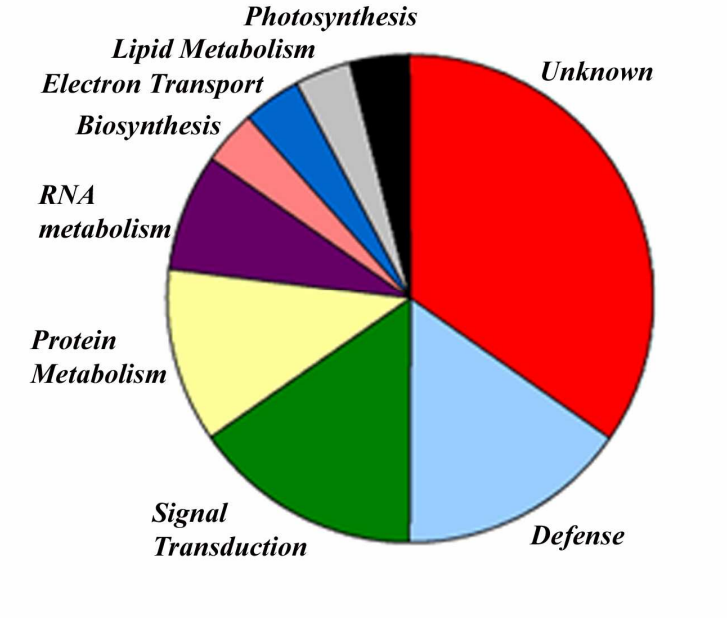

Figure 5

Functional classification of 25 genes for which VIGS altered the defense response *N. benthamiana*. Annotation was obtained by sequence comparison of the tobacco EST with *Solanacea* and *Arabidopsis thaliana* nucleotide databases.

Identification of genes that affect the defense response of tomato using VIGS

We used VIGS in tomato (*L. eculentum* VF36) to validate the results for the 25 selected genes (Table 5). A similar set-up as for the screen in *N. benthamiana* was followed in tomato. As it was shown that tobacco sequences could be used to silence the homologues tomato genes using the improved vector, pTRVRNA2-GW (Liu *et al*., 2002a), we cloned the 25 tobacco sequences in this vector.

VIGS constructs were agro-infiltrated into tomato plants (nine plants / construct) to induce gene silencing. After onset of the silencing symptoms in PDS infiltrated plants, two leaves of each infiltrated plant (position nine and ten, one being the oldest) were detached for pathogen assays. One leaf was used for infections with *Botrytis* and one for infection with *Sclerotinia* solutions. VIGS with 22 constructs did not alter the defense response of tomato against *Botrytis* or *Sclerotinia* (data not shown). VIGS of a 17kDa heat shock protein (HSP), a putative esterase/lipase and two mitogen-activated protein (MAP) kinase family proteins (NRK1 and NQK1), resulted in significantly increased sensitivity ($p < 0.05$) towards *Sclerotinia* infections (Table 6, Figure 6). In addition, spray infections with *Botrytis* showed that VIGS of NRK1 and NQK1 also resulted in increased sensitivity towards *Botrytis* (Figure 6). In tomato, disease symptoms from spray infections were classified with an arbitrary infectivity scale ranging from 0-3 (scale 0 = no lesions; scale 1 = only some small spots; scale 2 = 5 - 40 % affected leaf; scale 3 = more than 40% affected leaf) (Figure 3*B*).

The effect of NQK1 silencing on resistance against necrotrophic pathogens was confirmed in two independent experiments with 20 tomato plants. Although the results varied between the different experiments, we observed that NQK1-silenced plants showed significantly ($p < 0.05$) higher sensitivity to the pathogens than control plants (Figure 7), suggesting that NQK1 is involved in resistance against *Botrytis* and *Sclerotinia*. The other genes (HSP, NRK1 and the esterase/lipase) are still to be tested via independent experiments.

Table 6. Genes for which VIGS resulted in increased sensitivity of tomato to *Sclerotinia* infection.

ID	Annotation	*p*-value
NRK1	MAPK	0,035
NQK1	MAPKK	0,009
BC4-M34-045	Unkown, contains esterase/lipase thioesterase domain	0,002
BT2-M41-008	17 kDa HSP, CIII class	<0.001

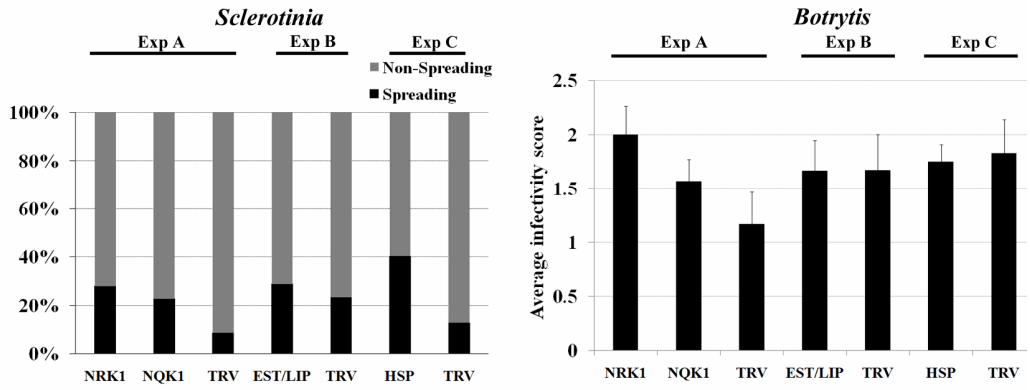

Figure 6

Results for *Sclerotinia* (left) and *Botrytis* (right) infections on tomato plants that were infiltrated with VIGS constructs for NRK1, NQK1, an unknown protein with esterase / lipase domain (EST/LIP) and small heat shock protein (HSP). In each experiment (Exp A, B, C), leaves from empty vector (TRV)-inoculated plants were used as controls. *Sclerotinia* infections were analyzed by classifying inoculation droplets as spreading and non-spreading lesions. *Botrytis* infections were scored via an infectivity scale ranging from 0 to 3 (n = 9).

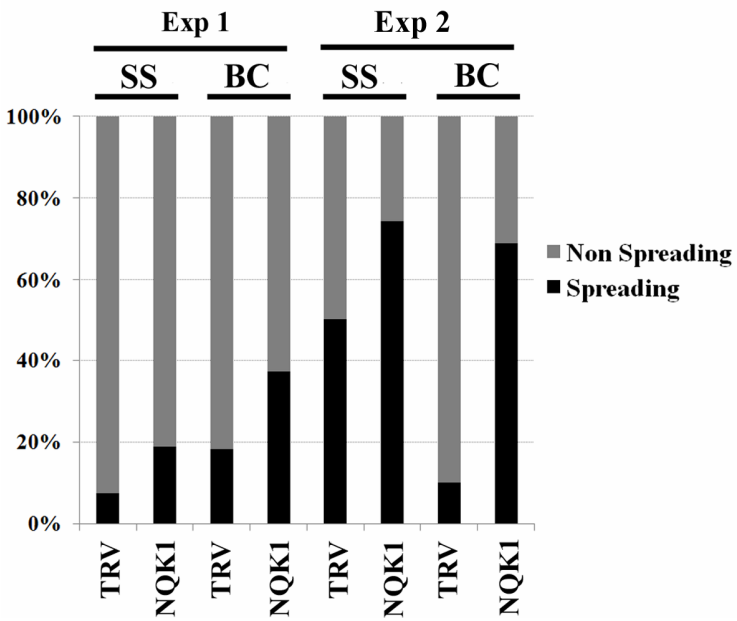

Figure 7

VIGS of NQK1 increases sensitivity of tomato against *Botrytis cinerea* (BC) and *Sclerotinia sclerotiorum* (SS). Results for two independent experiments (Exp 1, Exp 2) on 20 tomato plants are shown. One leaf of each plant was used for *Botrytis* infection and one was used for *Sclerotinia* infection.

DISCUSSION

ROS, such as H_2O_2, are associated with several aspects of the plant's defense response to pathogen infection, including transcriptional regulation of genes whose products help to defend the plant from pathogen attack (Lamb and Dixon, 1997). Necrotrophic pathogens, such as *Botrytis cinerea*, were shown to perturb the defense response of plants as an attack strategy (Lyon *et al*., 2004). Convincing evidence on the importance of H_2O_2 signaling in the defense response of plants against necrotrophic pathogens was delivered by Asselbergh and coworkers (2007), who showed that timely accumulation of H_2O_2 contributes to increased resistance of the tomato *sitiens* mutant and that this was associated with higher expression of known defense genes.

In this study, VIGS was used to investigate the participation of H_2O_2-induced genes in the defense response of plants against two necrotrophic pathogens, *Botrytis cinerea* and *Sclerotinia sclerotiorum*. Similar high-throughput approaches that combined transcriptome data with VIGS have proven to be successful for the discovery of genes that can function in defense of pepper (*Capsicum annuum*) against Tobacco Mosaic Virus and genes involved in *Pto*-mediated cell death in *N. benthamiana* (del Pozo *et al*., 2004; Kim *et al*., 2007). Tomato was chosen as host for our study because of its economical relevance since it is highly susceptible to *Botrytis* infection. We first evaluated the selected genes in *N. benthamiana* because this plant is more suitable for high-throughput manipulations and it can serve as a model for tomato (Lu *et al*., 2003). The TRV VIGS vectors that were used in this study belong to the most potent and widely used viral vectors and allows efficient VIGS in many different species, including *N. benthamiana* and tomato (*Lycopersicon esculentum*) (Liu *et al*., 2002a; Ratcliff *et al*., 2001). As heterologous gene sequences can be used to silence homologues sequences in related plant species (Senthil-Kumar *et al.,* 2007), it was not necessary to clone the tomato homologues of the tobacco genes.

We identified four genes (BYPASS, DRP, ThrRS, 26S RSU) for which VIGS resulted in phenotypic aberrations. As VIGS symptoms reflect the loss-of-function of the encoded protein, these genes must be important for normal plant growth and development. Moreover, the observation that VIGS of these genes led to phenotypic defects in both *N. benthamiana* and tomato indicates that these genes are probably involved in conserved biological pathways. Indeed, these genes are involved in transcription (DRP), translation (ThrRS) and protein degradation (26S RSU). The function of BYPASS is mostly unknown, but in *Arabidopsis*, BYPASS1 acts as a negative regulator of a mobile carotenoid-derived signal that is able to arrest plant growth (Van Norman *et al*., 2007). *Arabidopsis* BYPASS1 mutants are completely inhibited in shoot development, and this phenotype is similar as what we observed after VIGS in *N. benthamiana* (data not shown).

VIGS of four genes (encoding an HSP, an esterase, NRK1 and NQK1) increased sensitivity to *Sclerotinia* and/or *Botrytis*, which suggests that the encoded proteins are involved in the defense response against necrotrophic pathogens. Nothing is known on the specificity of the esterase/lipase protein, but it might be involved in cell wall modifications during disease resistance (Shah *et al*., 2005). HSPs, which act as molecular chaperones to protect protein function during abiotic stress, have also been implicated in plant disease resistance (Kanzaki *et al*., 2003; Lu *et al*., 2003; Hein *et al*., 2005; de la Fuente van Bentem *et al*., 2005; Scofield *et al*., 2005; Bhattarai *et al*., 2007).

Interestingly, NPK1 and NQK1 are both functional in the same mitogen-activated protein (MAP) kinase pathway (Figure 8). This pathway is designated as the tobacco NACK-PQR pathway, is evolutionary conserved with orthologues in at least yeast and *Arabidopsis,* and its main function is the regulation of cytokinesis (Soyano *et al*., 2002; Takahashi *et al*., 2004). The tobacco NACK-PQR pathway exists of an upstream MAP kinase kinase kinase (NPK1), a MAP kinase kinase (NQK1) and a downstream MAP kinase (NRK1), and can be activated by NACK1, which is a kinesin-like protein that co-localizes with NPK1 (Takahashi *et al*., 2004). In addition to a role during cytokinesis, evidence for a function during abiotic stress and hypersensitive response is also emerging, suggesting involvement of the NACK-PQR pathway in the interplay between cell cycle progression and stress responses (Hirt, 2000; Kovtun *et al*., 2000; Shou *et al*., 2004). Until now, the role of the NACK-PQR pathway during biotic stress has been exclusively studied via VIGS of NQK1, NPK1 and NRK1. It was shown that *N*-mediated resistance to Tobacco Mosaic Virus is attenuated in plants defective in NPK1, NQK1 and NRK1 (Jin *et al*., 2002; Liu *et al*., 2004). Moreover, VIGS of NPK1 interfered with function of *Bs2* and *Rx* during resistance against *X. campestris* and *P. syringae*, respectively, but it did not affect *Pto* and *Cf-4* mediated resistance (Jin *et al*., 2002; Leister *et al*., 2005). These data indicated that NPK1, NQK1 and NRK1 are involved in *R*/*Avr*-gene mediated hypersensitive cell death to confer resistance against avirulent pathogens. Accordingly, it was shown that NPK1 acts together with other MAP kinase cascades, involving MAPKKKα, MEK2, SIPK and WIPK, to induce cell death during pathogen attack (del Pozo *et al*., 2004).

CONCLUSIONS AND PERSPECTIVES

We have performed a VIGS screen for genes that affect the defense response of plants to *B. cinerea* or *S. sclerotiorum*. VIGS of four genes, encoding a mitogen-activated protein (MAP) kinase kinase kinase (NPK1), a MAP kinase kinase (NQK1), a heat shock protein (HSP) and a putative esterase / lipase protein, led to increased sensitivity to *Botrytis* and/or *Sclerotinia* in *N. benthamiana* and tomato. These genes might therefore be relevant candidates to increase the

resistance of plants against necrotrophic pathogens, but additional experiments will be needed to validate this hypothesis. The expression levels of the genes before and during infection need to be tested. However, this will require additional sequence information of regions outside the fragment used for VIGS. Since we can not exclude that VIGS led to silencing of non-target genes, an alternative approach would be to make stable tomato mutants.

In addition, VIGS of four H_2O_2-induced genes, encoding a BYPASS protein, a DRP, a ThrRS and a 26S RSU resulted in stunted plants with pleiotropic effects on normal plant development, indicating that these genes act in the interplay between H_2O_2 signaling and growth and development. The results for BYPASS are very relevant, especially because knock-out of BYPASS1 in *Arabidopsis* also affected shoot development (Van Norman *et al*., 2007).

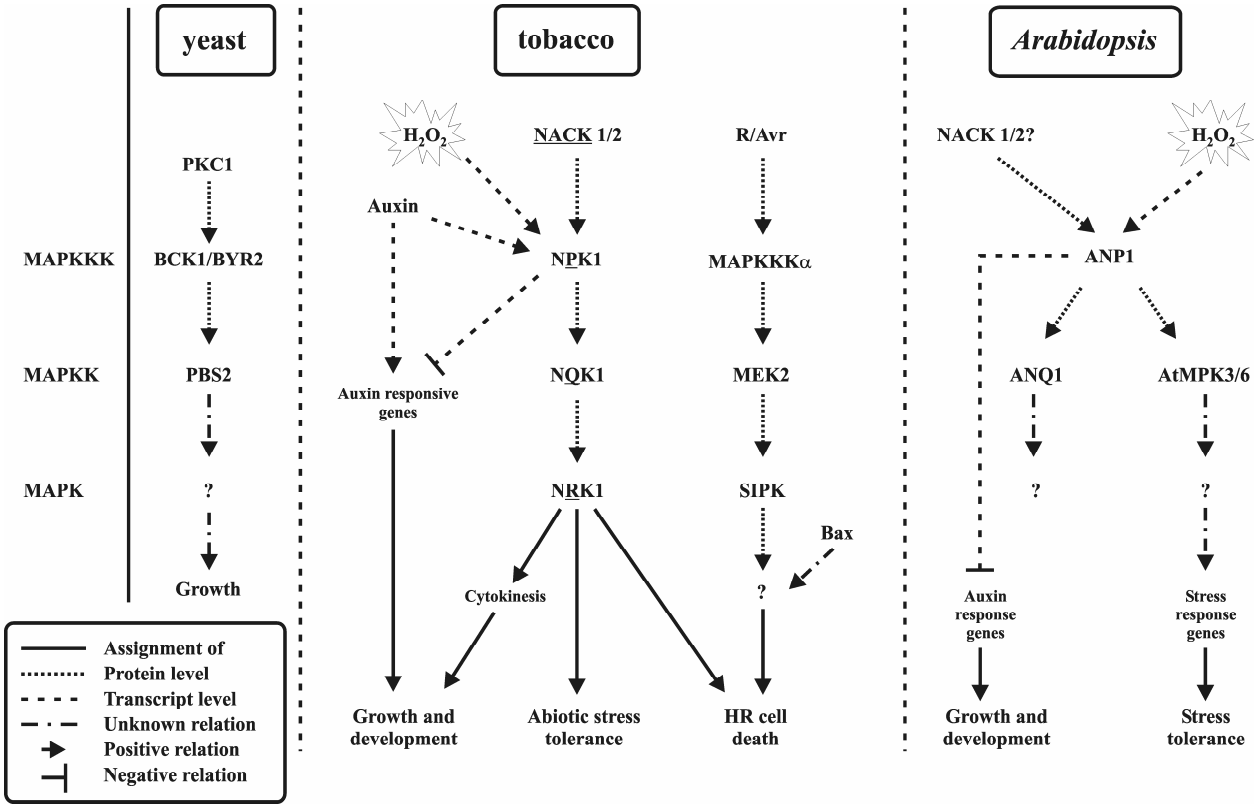

Figure 8

NACK-PQR pathway. The NACK-PQR pathways consist of an upstream kinesin-like protein and a downstream MAP kinase cascade involving NPK1, NQK1 and NRK1. This pathway is conserved in yeast and *Arabidopsis* and functions in growth and development, tolerance to abiotic stress and in hypersensitive response-mediated resistance against pathogens.

MATERIAL AND METHODS

Plant Material and Growth Conditions

N. benthamiana and tomato (*L. esculentum* VF36) plants were grown at 24 °C in a controlled growth chamber under a 16 h light /8 h dark cycle and 60% humidity. Tomato seeds were sterilized by incubation with 75 % ethanol for two minutes and 1% bleach for ten minutes and pre-incubated at 4 °C for one week before sowing. Seeds were soil-sown (saniflor) in plastic pots (Ø 12 cm) and kept under high humidity until germination. Two week old seedlings were transferred to individual pots (*N. benthamiana*, Ø 8 cm; tomato, Ø 16 cm).

Construction of TRV constructs

Isolated tobacco cDNA-AFLP fragments were reamplified in a PCR reaction using *Mse*0 (TCGATGAGTCCTGAGTAA) and *Bst*0 (CCGTAGACTGCGTAGTGATC) AFLP primers with *att*B1 (GGGGACAAGTTTGTACAAAAAAGCAGGCT) and *att*B2 (GGGGACCACTTTGTACAAGAAAGCTGGGT) adaptor sequences, respectively, to make them compatible for Gateway® recombinatorial cloning. The resulting PCR fragments were cloned into pDONR207 via site-specific recombination between *att*B and *att*P sequences, and transferred into competent *E. coli* DH5α cells by heat shock transformation. Independent colonies were tested via PCR with DNR3 (GATGGTCGGAAGAGGCATAA) and DNR5 (CTGGCAGTTCCCTACTCTCG) primers which were designed against regions on the backbone of the pDONR207 vector so that the corresponding PCR product contained the pDONR207 *att*L1 and *att*L2 recombination sites flanking the cDNA-AFLP fragment. The resulting PCR products were directly used in a site-specific recombination reaction with the *att*R sites of the destination vector, either being pTV00::GW2 or pTRVRNA2-GW for VIGS in *N. benthamiana* and in tomato, respectively.

pTV00::GW2 was generated from the original pTV00 vector by subcloning a 1756 bp *SpeI-ApaI* DNA fragment containing the gateway® GW2 cassette from pGW2 (http://www.psb.ugent.be/gateway) into the multi cloning site of pTV00 in order to make it compatible for the Gateway® cloning technology (Invitrogen Corp., Carlsbad, CA, USA). The resulting vector contains *attR* sites and the *ccd*B gene and was designated pTV00::GW2.

The *att*L/*att*R reaction mixture was then transferred into competent *E. coli* DH5α cells by heat shock transformation and positive clones were identified using colony PCR with *att*B1 and *att*B2 primers. Plasmids were isolated from positive clones using the Wizard® Magnesil® plasmid purification System from Promega (cat nr. A1631), transferred into *Agrobacterium tumefaciens strain* GV3101 by freeze-thaw positive clones were identified using colony PCR with *att*B1 and *att*B2 primers.

Virus-Induced Gene Silencing

Virus infections on *N. benthamiana* were achieved by *Agrobacterium*-mediated transient gene expression of infectious constructs from the T-DNA of the binary vector pTV00::GW2. Before VIGS infection, pTV00::GW2 was mixed with a helper plasmid for replication, pBINTRA6, which contains a full-length infectious cDNA clone of TRV RNA1 (Ratcliff *et al*., 2001). *Agrobacterium* cultures containing pTV00::GW2-derived and pBINTRA6 vectors were grown in appropriate selection medium until saturation. Bacterial pellets were resuspended in infiltration solution, containing 100 µM acetosyringone (3,5-dimethoxy-4-hydroxyacetophenone), 10 mM $MgCl_2$ and 10 mM MES pH5.6 until an OD_{600} of 1.0. pBINTRA6 and pTV00::GW2 suspensions were incubated at room temperature for 3 h, mixed in a 1:1 ratio and infiltrated with a needle less syringe in the underside of the third and fourth leaf of 2-3 week old-plants. For VIGS in tomato, pTRVRNA1 and pTRVRNA2-GW-derived vectors were used (Liu *et al*., 2002). The same infiltration method was applied as in *N. benthamiana*, with the single modification that the bacterial pellets were diluted to an OD_{600} of 2.0.

Fungal material and infection method

Conidia of *Botrytis cinerea* strain R16 (Faretra and Pollastro, 1991) were obtained as described by Audenaert *et al*. (2002). Briefly, a *Botrytis* plaque is grown on Potato Dextrose Agar (PDA) medium (Difco™) at 22°C for circa five days until full coverage with mycelium and placed under ultraviolet light (12 h dark /12 h UV light cycle; PHILIPS 18W/08 and PHILIPS TLD 18W/33 light source) for ten extra days to induce sporulation. The conidial suspension was filter and centrifuged for ten min at 10000 g. After removal of the supernatant and resuspension of the conidia in distilled water, an inoculation suspension was prepared containing 6.25 x 10^4 spores / ml, 6.67 mM KH_2PO_4 and 0.01 M glucose. Conidia were pre-germinated for two h in the inoculation suspension at 22 °C prior to infection. *Sclerotinia sclerotiorum* was grown on PDA medium (Difco™) at 18 °C at 22 °C for circa five days until full coverage with mycelium. Liquid cultures were obtained by growing mycelium plaques for one to two weeks in Roux flasks containing 100 ml Potato Dextrose Broth (Difco™) medium. For infections, the mycelium was removed from the medium, washed with sterile water. The mycelium suspension was homogenized in sterile water with an electronic mixer (IKA®-WERKE) and diluted to an OD_{595} of 1.0 for infection.

All infections were done on detached leaves. The leaves were arranged on Petri dishes in plastic trays containing 200 ml of water and two layers of absorbed paper in such way that only the petioles were in contact with the wet paper. A piece of wet paper was put on the petioles to improve contacted with the wet paper in the tray. For drop infection assays, five µl droplets were

used to inoculate each leaf. Spray infection of *Botrytis* were done with a perfumer so that the leaves were covered with droplets of 1-2 µl. The trays containing the leaves were covered with plastic paper to obtain high humidity and incubated at 18 or 22 °C under dark conditions. Symptoms were evaluated after 4-5 days. Each inoculation droplet was classified as a spreading or non-spreading lesion. The data were statistically analyzed with a binary logistic regression using SPSS software. Spray inoculations were digitally scored using APS Assess (Image Analysis Software for Plant Disease Quantification by Lakhdar Lamari, APS press) or scored via an arbitrary infectivity scale ranging from 0 to 3, 0 being no disease symptoms, 1 if only small spots are observed, 2 if less that 40 % of the leaf is infected and 3 representing almost complete (> 40 %) coverage of the leaf with disease symptoms.

Supplementary Tables

Supplementary Tables for this chapter are included as addendum at the back of thesis and are also made available in an electronic version on the attached compact disc.

Supplementary Table S4. Selected cDNA-AFLP fragments

ACKNOWLEDGMENTS

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CHAPTER 4

Ectopic expression of the WRKY15 transcription factor in *Arabidopsis* increases tolerance to oxidative stress

ABSTRACT

Reactive oxygen species (ROS) are important signal molecules in plant defense responses to environmental stress conditions. Accumulation of ROS, including H_2O_2, during abiotic stress leads to the transcriptional induction of genes that encode proteins involved in cellular protection, and regulatory genes encoding signaling proteins and transcription factors (TFs). We screened 12 H_2O_2-induced regulatory genes for their potential to induce stress tolerance when constitutively expressed in *Arabidopsis*. Ectopic expression of WRKY15 (WRKY15^{OE}), which belongs to a large divergent family of plant-specific TFs, leads to increased rosette size and tolerance to H_2O_2. Moreover, WRKY15^{OE} transgenic lines were more tolerant to osmotic stress, but more sensitive to salt stress. Co-expression analysis suggests that WRKY15 acts as a downstream component of a MAPK signaling pathway that is part of the oxidative stress signal transduction network of *Arabidopsis*. Our data suggests an important function for WRKY15 during H_2O_2 signal transduction and the defense response of plants to abiotic stress.

INTRODUCTION

ROS were originally considered as toxic byproducts of oxygen metabolism and their production is increased during environmental stress, including low/high temperatures, drought, salt, heavy metal, high light, ultraviolet radiation and air pollution (Apel and Hirt, 2004). Accumulation of ROS during such stresses can result in extensive cellular damage, which is referred to as oxidative stress (Halliwell, 2006). Transgenic plants that can tolerate oxidative stress often possess broad spectrum stress resistance (Ahmad *et al*., 2007; Kasukabe *et al*., 2004; Tang *et al*., 2007a; Tognetti *et al*., 2006). At lower concentrations, ROS can act as secondary messengers by controlling the expression of genes of which the encoded proteins are important for plant growth and development, including adaptation to stress. (Dat *et al*., 2000; Gechev *et al*., 2006).

Recent studies in *Arabidopsis* have revealed key components involved in the ROS signal transduction network of plants (Mittler *et al*., 2004). ROS can activate protein kinases, such as ANP1 (*Arabidopsis* NPK1-like protein 1), MAPK3/6 (mitogen-activated protein kinase 3/6), OXI1 (oxidative stress inducible 1), PDK1 (phosphoinositide-dependent kinase 1), PTI1-2 (*Pto* kinase interactor 1) and NDPK2 (nucleotide diphosphate kinase 2), and can also induce alterations in calcium fluxes and other biochemical changes (Anthony *et al*., 2006; Kovtun *et al*., 2000; Moon *et al*., 2003; Rentel *et al*., 2004a; Rentel *et al*., 2004b). TFs are important regulators within the ROS signal transduction network. The expression of one third of all TFs (approximately 1500 TF exist in *Arabidopsis*) is induced by ROS, and some of them, including heat shock factors and members of WRKY, MYB, ZAT, and RAV families are involved in the stress response of plants (Gadjev *et al*., 2006; Mittler *et al*., 2004).

The production of transgenic *Arabidopsis* plants with compromised levels of specific antioxidant enzymes, together with the advent of genome-wide microarrays have enabled researchers to analyze the transcriptional changes caused by increased ROS levels (Rizhsky *et al*., 2004; Davletova *et al*., 2005; Umbach *et al*., 2005; Vandenabeele *et al*., 2004; Vanderauwera *et al*., 2005). Comparison of such datasets revealed that the cellular response depends of the specificity of the ROS signal as well as the cellular site of production (Gadjev *et al*., 2006). Plants with decreased levels of catalases, which are the main H_2O_2-scavenging enzymes in the peroxisomes of plants, were used as a model system to specifically study the signaling role of photorespiratory H_2O_2 (Vandenabeele *et al*., 2004; Vanderauwera *et al*., 2005). High light treatment of plants increases photorespiration and production of glycolate, which is converted to glyoxalate and H_2O_2 in the peroxisomes. By exposing catalase-deficient plants to high light, endogenous H_2O_2 levels can be modified to study its impact on gene expression. Genome-wide microarray analysis of the transcriptional changes that occur in catalase-deficient plants exposed to high light resulted in the

identification of 437 H_2O_2-regulated genes, of which 80% was induced. In addition, it was shown that a relevant number of H_2O_2-upregulated genes were also induced by cold, heat or drought, suggesting that they act in multiple defense responses against abiotic stresses (Vanderauwera *et al*., 2005). A more detailed analysis is necessary to clarify the function of these H_2O_2-induced genes in the ROS network of plants. Mutation or ectopic expression of several of the genes that were induced by H_2O_2 in catalase-deficient plants was already shown to provide tolerance to abiotic stresses, including oxidative stress and heat stress (Table 1).

The aim of this work was to screen *Arabidopsis* H_2O_2-induced genes for a possible role during stress tolerance. An initial selection of *Arabidopsis* H_2O_2-induced genes, identified using an *Arabidopsis* 6K cDNA microarray (Vandenabeele *et al*., 2004), was made based on the presence of H_2O_2-responsive tobacco homologues that were identified in an earlier study (Vandenabeele *et al*., 2003). Additional selection criteria included temporal expression pattern (early induction by H_2O_2) and sequence characteristics such as the presence of defined regulatory protein domains (Vanderauwera, 2007). Because of the current interest of our research group in the molecular networks steering H_2O_2 signaling, the focus is on TFs and other regulatory genes. Transgenic lines for 12 such H_2O_2-induced regulatory genes (listed in Table 2) were screened for increased tolerance or sensitivity to oxidative stress and heat stress. Figure 1 shows the H_2O_2-induced expression of the selected genes after high light exposure of catalase-deficient plants.

Ectopic expression of the WRKY15 TF in *Arabidopsis*, increased leaf size and resulted increased tolerance to oxidative and osmotic stress, but also increased sensitivity to salt stress.

Table 1. Genes that were induced by high light treatment of catalase-deficient *Arabidopsis* plants (H_2O_2-induced genes) and were shown to increase abiotic stress tolerance

Gene	AGI	Description	FC[1]	Reference
Tolerance to oxidative stress				
LEA5	AT4G02380	late embryogenesis abundant 5	6.0	Mowla *et al*., 2006
GLB1	AT2G16060	non-symbiotic hemoglobin	3.1	Yang *et al*., 2005
BCB	AT5G20230	blue copper-binding protein	2.9	Ezaki *et al*., 2000
Tolerance to heat stress				
HSP101	AT1G74310	heat shock protein 101	22.2	Queitsch *et al*., 2000
DREB2A[2]	AT5G05410	drought-responsive element binding protein 2A	8.0	Sakuma *et al*., 2006b
MBF1C[2]	AT3G24500	multi-bridge binding factor 1C	7.0	Suzuki *et al*., 2005
Tolerance to oxidative and heat stress				
HSFA2[2]	AT2G26150	heat shock factor A2	27.6	Ogawa *et al*., 2007
RHL41[2]	AT5G59820	responsive to high light 41	3.3	Davletova *et al*., 2005
Tolerance to other abiotic stresses				
HSP17.6A	AT5G12030	heat shock protein 17.6 kDa	9.3	Sun *et al*., 2001
CBL1	AT4G17615	calcineurin B-like protein	3.9	Cheong *et al*., 2003; Albrecht *et al*., 2003
AOX1[2]	AT3G22370	alternative oxidase	2.4	Fiorani *et al*., 2005;Umbach *et al*., 2005

[1] Fold change induction after 3h high light treatment (Vanderauwera *et al*., 2005); [2] Molecular phenotype available

Table 2. Overview of selected H_2O_2-induced *Arabidopsis* genes

AGI	Gene	Functional data in literature	Reference
Transcription Factors			
AT1G01720	NAC domain protein (ATAF1)	Negative regulator of drought stress	Lu *et al*., 2007
AT2G23320	WRKY15		
AT2G38470	WRKY33	Negative regulator of salycilic acid-dependent defense against and positive regulator of jasmonic acid / ethylene-dependent defense responses	Andreasson *et al*., 2005; Zheng *et al*., 2006;
AT3G29035	NAM like protein (ANAC059)		
AT3G54620	bZIP TF-like (AtbZIP 25)		
AT5G05410[1]	DREB2A	Positive regulator of drought and heat stress	Sakuma *et al*., 2006a; Sakuma *et al*., 2006b
Protein kinases			
AT4G01370[1]	MAP kinase 4 (MPK4)	Protein kinase involved in H_2O_2 signal transduction and abiotic and biotic defense responses of plants	Peterson *et al*., 2000; Andreasson *et al*., 2005; Broderson *et al*., 2006: Nakagami *et al*., 2006
AT4G24400	Ser/Thr kinase like protein (CIPK 8)	Possible role in sugar signaling	Gong *et al*., 2002
Other functional class			
AT4G31920	Predicted protein (ARR10)	Possible positive regulator of cytokinin signal transduction	Mason *et al*., 2005
AT1G33600	Unknown protein (LRR domain)		
AT1G56450	20S proteasome beta subunit PBG1		
AT1G20580	expressed protein, snRNP domain		

[1] Molecular phenotype available

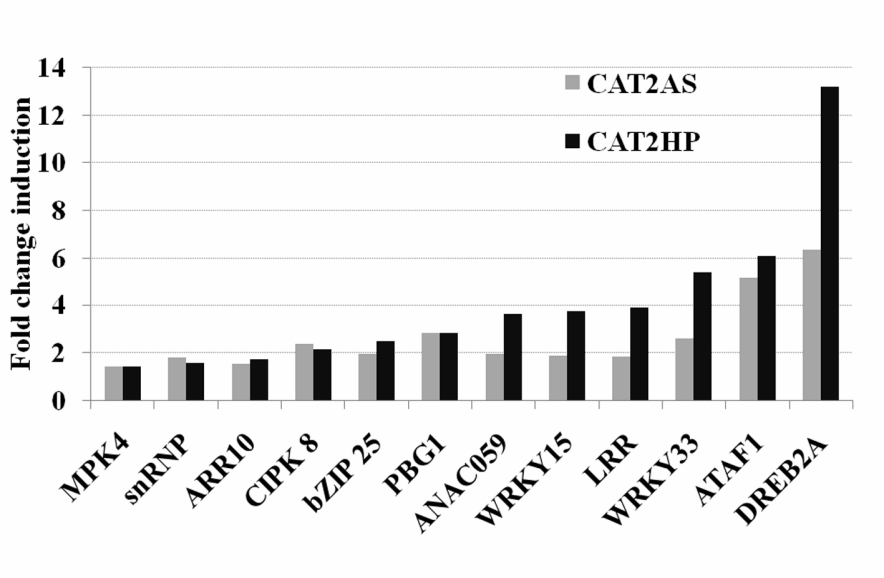

Figure 1

H_2O_2-induced expression of the selected genes. H_2O_2-induced genes were identified by high light exposure of catalase-deficient plants. The fold change induction compared to control plants is shown. CAT2AS and CAT2HP plants have 65 % and 20 % residual catalase activity, respectively.

RESULTS

Production of transgenic lines with increased levels of H_2O_2-induced genes

The open reading frames of the genes from Table 2 were amplified and introduced into the overexpression vector pB7WG2D using the Gateway® recombination system, (Karimi *et al*., 2002). The obtained overexpression constructs were transformed into wild type (WT) *Arabidopsis* (Col-4) plants by *Agrobacterium*-mediated floral dip transformation (Clough and Bent, 1998), and for each overexpression line, two independent transformants with a single T-DNA transgene insertion and enhanced transgene mRNA abundance were selected for the production of homozygous lines and further functional analysis.

Optimization of stress assays: Analysis of WT plants exposed to oxidative and heat stress

To select an appropriate concentration for the oxidative stress screen, WT plants were first grown *in vitro* on different H_2O_2 concentrations (0 mM, 0.5 mM, 1.0 mM, 1.5 mM, 2 mM, 5mM) for two weeks and checked for germination capacity and seedling development. Figure 2 shows that H_2O_2 concentrations of 0.5 mM had no negative effect on the rosette phenotype. In contrast, treatment with 1 mM H_2O_2 resulted in stunted plants and leaf yellowing. Treatment with 2 mM H_2O_2 resulted in complete chlorosis and inhibition of the development of rosette leaves. No effect on germination was observed for concentrations lower than 2 mM, while 5 mM H_2O_2 inhibited seed germination. Because of our interest in the effect of oxidative stress on plant growth rather than germination, 1 mM H_2O_2 was chosen as the optimal concentration for assessing tolerance to oxidative stress. In further experiments, plants were scored as healthy, damaged (chlorotic, yellow and/or retarded in growth but still viable) and dead (no leaf development) (see Figure 2 for phenotypes).

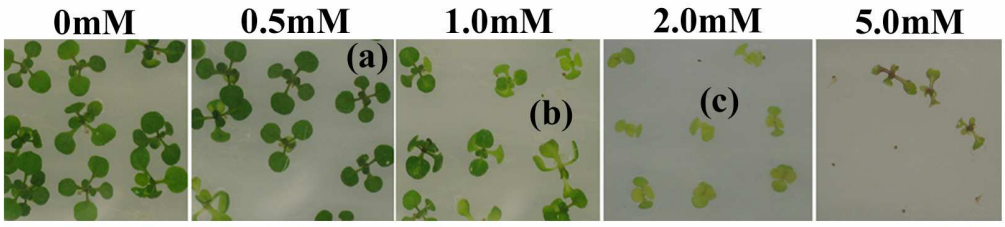

Figure 2

Phenotype of WT plants exposed to different H_2O_2 concentration. Photographs were taken ten days after germination. Phenotypes of healthy (a), damaged (b) and dead (c) plants is indicated.

Next, we studied the dose-response of WT plants to high temperatures (heat stress). Heat tolerant transgenic plants overexpressing HSP101 ($HSP101^{OE}$) were used as positive control (Queitsch *et al*., 2000). One week-old WT and $HSP101^{OE}$ seedlings were exposed to 38 °C for 3, 6, 9, 12, 24 and 48 h and were scored after 1, 2, 3 and 7 days recovery at normal temperatures. Exposure for 3 h and 6 h at 38 °C did not result in any visible effect (data not shown). Exposure for 9 h affected 10-20 % of the seedlings, but the seedlings recovered from the treatment and produced new and green leaves (data not shown). In contrast, 40 % of the WT seedlings that were exposed to a 12 h heat shock were damaged or dead (see Figure 2 for phenotypes), and longer exposure times (24 h or 48 h) completely killed almost all seedlings (Figure 3*A*).

$HSP101^{OE}$ seedlings were more tolerant than WT seedlings after a 12 h treatment (Figure 3*A*, 3*B*). Since seedlings that were damaged by 12 h exposure at 38 °C could not completely recover from it, this treatment was chosen for further evaluation of plants for heat stress tolerance. Because overexpression of DREB2A in *Arabidopsis* and other plant species was previously reported to enhance tolerance to heat stress (Sakuma *et al*., 2006b; Qin *et al*., 2007), we first tested the chosen heat stress treatment on transgenic lines that constitutively express DREB2A ($DREB2A^{OE}$). Our data confirmed the increased heat tolerance of $DREB2A^{OE}$ seedlings (Figure 3*C*, 3*D*). Taken together, we were able to confirm the heat tolerance of HSP101 and DREB2A transgenic plants, which indicates the robustness of the assay.

Phenotyping of transgenic lines with perturbed levels of H_2O_2-induced genes under control conditions

Transgenic lines were first grown *in vitro* together with non-transformed control plants (WT Col-4) to compare their phenotype under non-stressed conditions. Per gene, one experiment on 40 WT seedlings and seedlings from two independent overexpression lines was performed. Plants were scored for the number of healthy (green) and damaged (yellow or chlorotic) plants (see Figure 2 for phenotypes of such plants). For WT, over 90 % of all plants were healthy. Transgenic plants overexpressing LRR (LRR^{OE}), WRKY33 ($WRKY33^{OE}$), ATAF1 ($ATAF1^{OE}$) and ANAC059 ($ANAC059^{OE}$) were more chlorotic and retarded in growth (< 80 % healthy plants) than WT (Figure 4*A*). Ectopic expression of snRNP ($snRNP^{OE}$) did not result in leaf yellowing or chlorosis, but in smaller plants with an altered leaf shape. This phenotype of $snRNP^{OE}$ plants was even more pronounced when exposed to stress or when expressed in the catalase-deficient background, both resulting in drastic leaf narrowing (Vanderauwera, 2007; Figure 4*B*).

The growth reduction was quantified by measuring the total rosette area of two week-old plants. For LRR^{OE}, $WRKY33^{OE}$, $ATAF1^{OE}$, $ANAC059^{OE}$ and $snRNP^{OE}$ plants, a decrease in rosette area

(10-30 %) compared to WT plants was observed (Figure 4*C*). We conclude that overexpression of LRR, WRKY33, ATAF1, ANAC059 and snRNP inflicts a yield penalty on plants and these genes were therefore excluded for further analysis. The decreased rosette area (and increased chlorosis) of ATAF1^{OE} lines was confirmed in an independent experiment (data not shown). The experiments for the other transgenic lines were not repeated. In contrast, ectopic expression of one H_2O_2-induced gene, encoding a WRKY15 TF, resulted in increased total rosette area (Figure 4*C*).

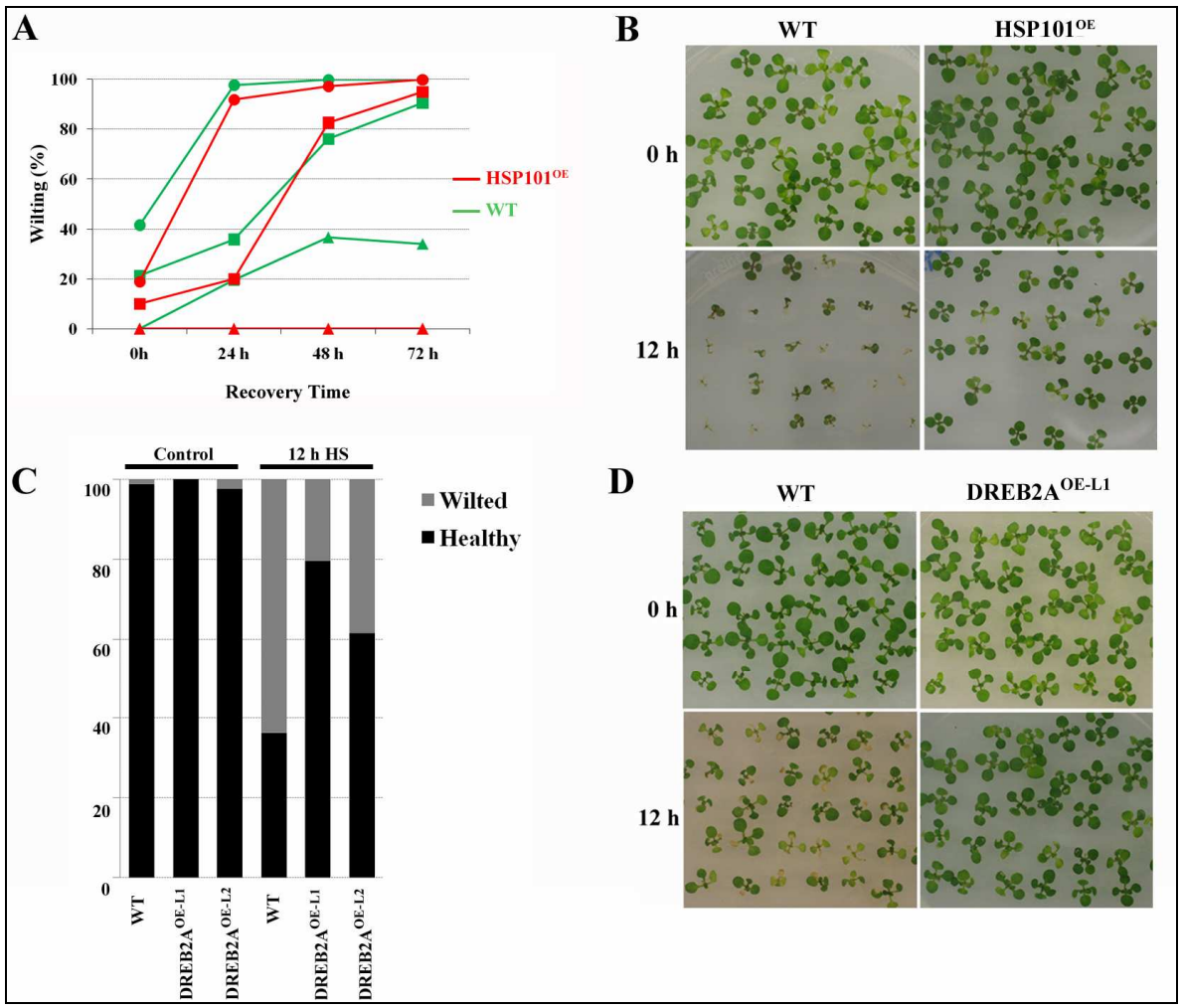

Figure 3

Heat tolerance of HSP101^{OE} and DREB2A^{OE} transgenic lines. Wilted plants include both damaged and dead plants (see Figure 2 for phenotypes). **A** Dose response curve of WT and HSP101^{OE} transgenic plants exposed to a heat shock of 38°C (triangles, squares and circles represent 12 h, 24 h and 48 h heat shock, respectively). **B** Phenotype of WT and HSP101^{OE} under control conditions (0 h) and after 12 h heat shock at 38 °C. **C** Heat stress tolerance of DREB2A^{OE} transgenic lines. **D** Phenotype of WT and DREB2A^{OE-L1} under control conditions (0 h) and after 12 h heat shock at 38 °C.

Screening of transgenic lines with increased levels of H_2O_2-induced genes for altered resistance to oxidative and heat stress

Transgenic lines with increased levels of H_2O_2-induced genes were screened for increased tolerance or sensitivity to oxidative stress. Per gene, one experiment with 40 WT seedlings and 40 seedlings from two independent transgenic was performed. Seedlings were grown for two weeks on medium containing 1 mM H_2O_2 and scored as healthy, damaged or dead (see Figure 2 for phenotypes of such plants). No relevant differences compared to WT plants were found for CIPK8^{OE}, bZIP25^{OE}, PBG1^{OE} and MPK4^{OE} transgenic plants (data not shown). In contrast, one ARR10^{OE} transgenic line displayed 20% more healthy plants than WT when grown on 1 mM H_2O_2, indicating increased tolerance to oxidative stress. The strongest increased tolerance was observed for the two WRKY15^{OE} transgenic lines, which displayed 20 and 70 % more healthy plants on 1 mM H_2O_2 compared to WT (Figure 5*A*, 5*B*).

Transgenic lines with increased levels of H_2O_2-induced genes were also screened for increased sensitivity or tolerance to heat stress. Per gene, one experiment with 40 WT seedlings and 40 seedlings from two independent transgenic lines was performed. Seedlings were grown for 1 week under normal temperatures, transferred to 38 °C for 12 h and allowed to recover for one week at normal temperatures. Plants were scored as healthy, damaged or dead (see Figure 2 for phenotypes of such plants). No relevant differences in heat tolerance were found for CIPK8^{OE}, PBG1^{OE}, bZIP25^{OE} and WRKY15^{OE} plants (data not shown). One MPK4^{OE} line and both ARR10^{OE} lines showed 10-20% more healthy plants after the heat shock (Figure 5*C*, 5*D*).

Of all tested transgenic lines, the increased resistance of WRKY15^{OE} plants to H_2O_2-induced oxidative stress was most relevant and these were therefore selected for further analysis.

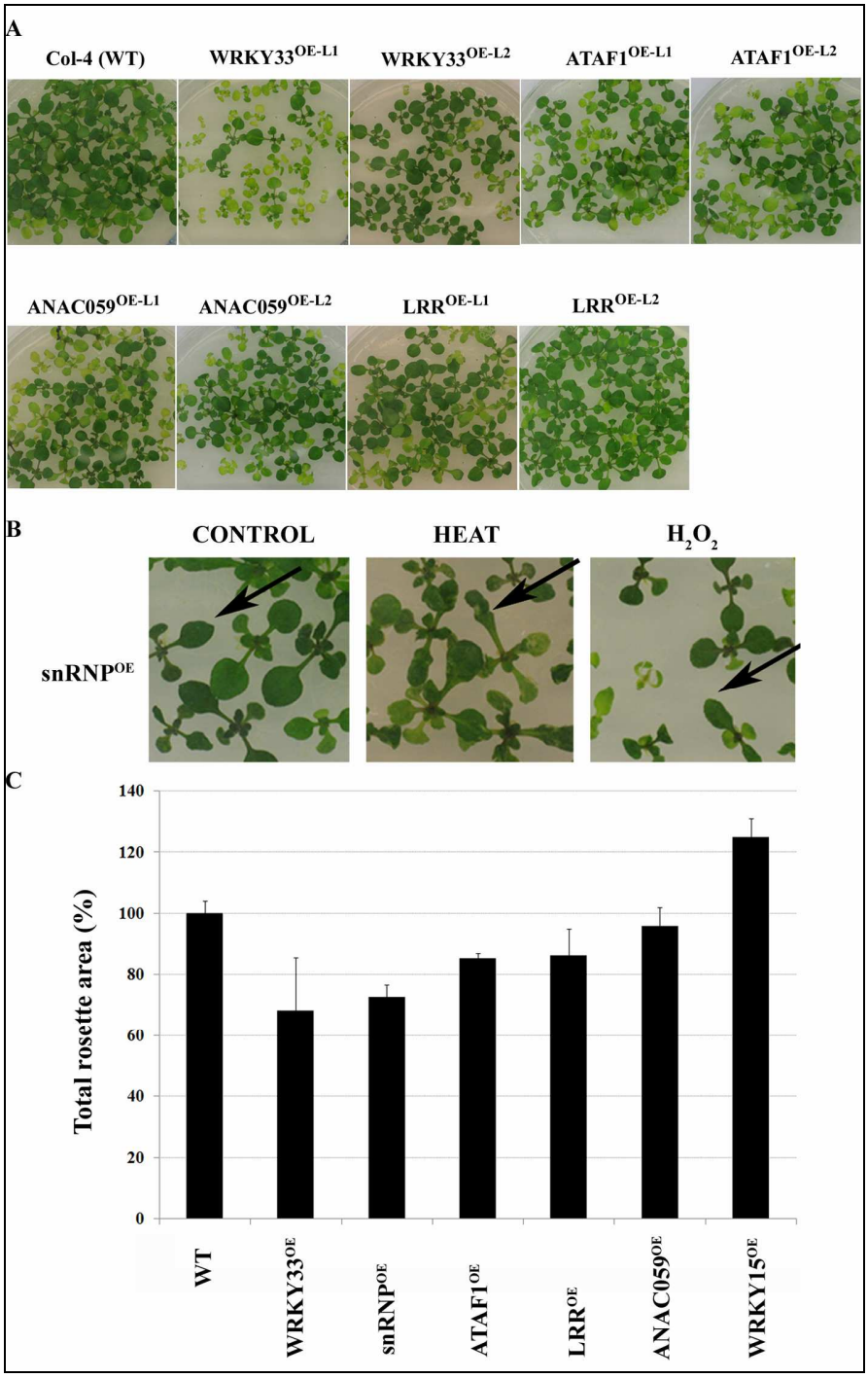

Figure 4

Phenotypes of H_2O_2-transgenic lines. **A** Phenotypes of transgenic lines that were visually scored as being more sensitive. **B** Phenotype of snRNP^{OE} under control, heat and H_2O_2 stress conditions. See figures 2 and 3 for the phenotype of WT plants H_2O_2 and heat stress conditions. Arrows indicate narrow leaves. **C** Quantification of total rosette area. For transgenic lines, the mean on two independent transformants is shown. For wild type, n = 13. (error bars represent SE).

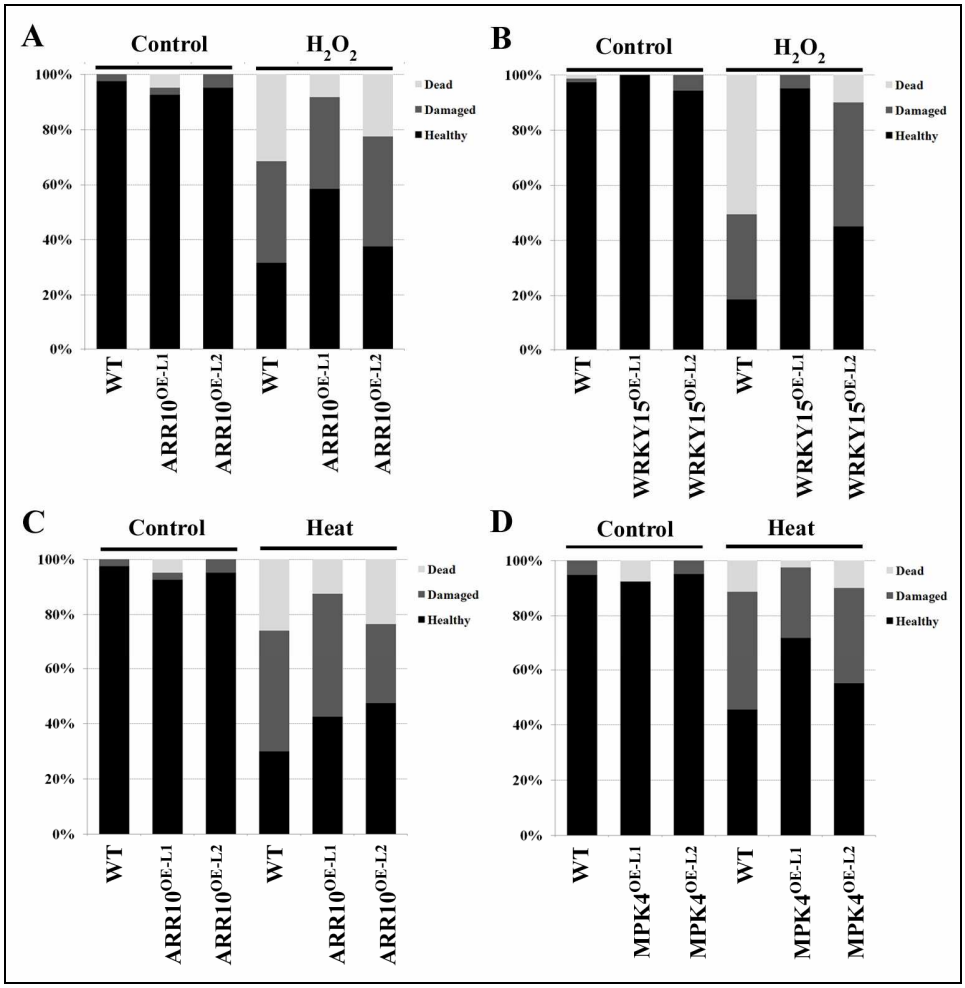

Figure 5

Stress tolerance of transgenic lines with perturbed levels of H_2O_2 genes. For each line, 40 seedlings were grown *in vitro* for two weeks on 1 mM H_2O_2, or grown on growth medium for 1 week and then transferred to 38°C for 12 h (heat). Growth plates were scored for seedlings that look healthy, stressed or dead (see Figure 2 for phenotypes of such plants). Results are shown for two independent lines per genotype. For WT, results are the average of two replicates. **A** Stress tolerance of $ARR10^{OE}$ plants to H_2O_2. **B** Stress tolerance of $WRKY15^{OE}$ plants to H_2O_2. **C** Heat stress tolerance of $ARR10^{OE}$ plants. **D** Heat stress tolerance of $MPK4^{OE}$ plants to H_2O_2.

Ectopic expression of WRKY15 increases tolerance to oxidative stress

Our results indicated that ectopic expression of WRKY15 increased rosette area of plants under control conditions and led to an increased tolerance to H_2O_2-induced oxidative stress *in vitro*. To confirm the tolerance of WRKY15^{OE} plants to H_2O_2, we first repeated the experiment from the screen. Per genotype (WT and WRKY15$^{OE\text{-}L1}$), 30 plants were grown (in duplicate) for two weeks on growth medium containing different H_2O_2 concentrations (0.0, 0.5, 1.0 and 1.5 mM H_2O_2). The phenotype of the plants was digitally scored and the effect of H_2O_2 on total rosette area was quantified (see Materials and Methods for more details). As before, it was observed that WRKY15^{OE} plants performed better compared to WT plants, especially when grown on medium containing H_2O_2 (Figure 6*A*). To further quantify the increased rosette size of WRKY15^{OE} plants, complete leaf series of 23 days old plants grown on growth medium containing different H_2O_2 concentrations (0.0, 0.5, 1.0 and 1.5 mM H_2O_2) were made. No significant increase in total leaf area was observed under control conditions (0.0 mM H_2O_2), but analysis of individual leaf area showed that leaves 3 to 8 of WRKY15^{OE} plants were larger than those of WT plants, while younger leaves were smaller or even not developed. High H_2O_2 concentrations (1.0 and 1.5 mM) increased the total leaf area with more than 25 % (Figure 6*B*). Individual leaf area of WT plants was more affected by 1.5 mM H_2O_2 than that of WRKY15^{OE} plants (Figure 6*C*). We conclude that ectopic expression of WRKY15 increases the tolerance of plants to H_2O_2-induced oxidative stress.

WRKY15 is involved in the response of plants to abiotic stress

We next addressed whether WRKY15 is involved in de response of plants to abiotic stress. To this end, WRKY15^{OE} transgenic plants were assayed for tolerance to salt, osmotic, cold and heat stress *in vitro* (see materials and methods for more details). To score the stress tolerance, the survival rate of WRKY15^{OE} and WT plants was determined. Plants developing true leaves were designated as survivors (see Figure 7*A* for a representative picture). We did not observe differences in survival rates to cold, heat and osmotic stress between WRKY15^{OE} and WT plants (data not shown). In contrast, quantitative analysis of seedling survivors on 100 mM NaCl indicated a survival rate of 60 % for WT seedlings, whereas only 10-20 % of WRKY15^{OE} seedlings survived this treatment (Figure 7B). Biomass (determined as dry weight) of WRKY15^{OE} plants grown on 100 mM NaCl was significantly lower (ca 30 %) than that of WT plants (Figure 7C). Although no difference between the survival rate of WRKY15^{OE} and WT plants was observed on 50 mM mannitol, we observed that total rosette area of WRKY15^{OE} plants on 50 mM mannitol was higher than that of WT plants and this resulted in an approximately 30% increase in plant biomass (Figure 7C). We also performed root

growth measurements on different concentrations of NaCl or mannitol, but these analysis revealed no significant differences between WRKY15^{OE} and WT plants (data not shown).

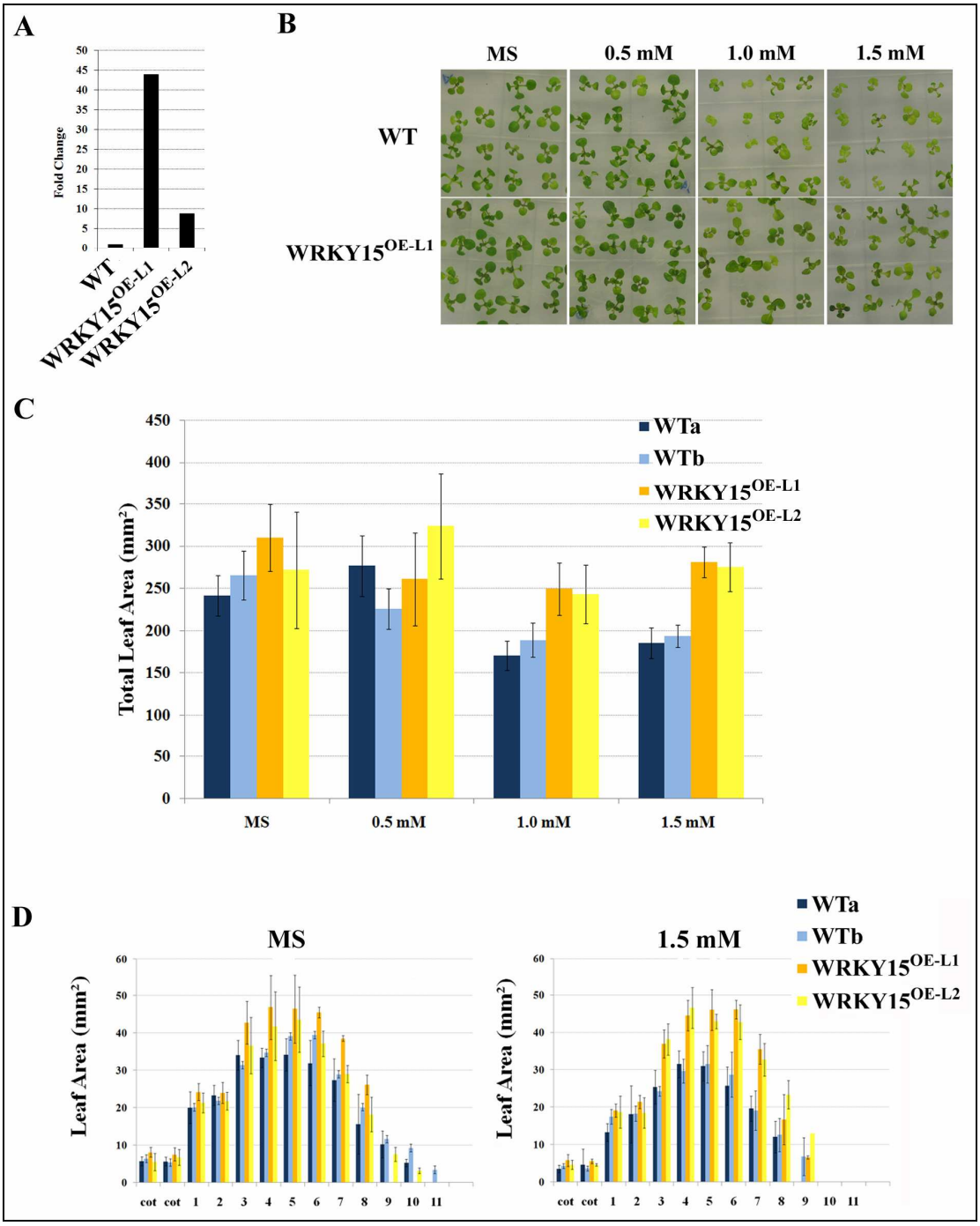

Figure 6

Increased tolerance of WRKY15^{OE} plants to H_2O_2. **A** Expression levels of WRKY15 transgene in two independent transformants, WRKY15$^{OE\text{-}L1}$ and WRKY15$^{OE\text{-}L2}$. **B** Phenotype of WT (Col-4) and WRKY15^{OE} plants when grown on growth medium supplemented with 0, 0.5, 1.0 and 1.5 mM H_2O_2. **C** Total leaf area of 23 days-old plants (n=4, error bars are standard deviation). **D** Individual leaf area of 23 days-old plants (n=4, error bars are standard deviation). WTa and WTb represent two different seed stocks,

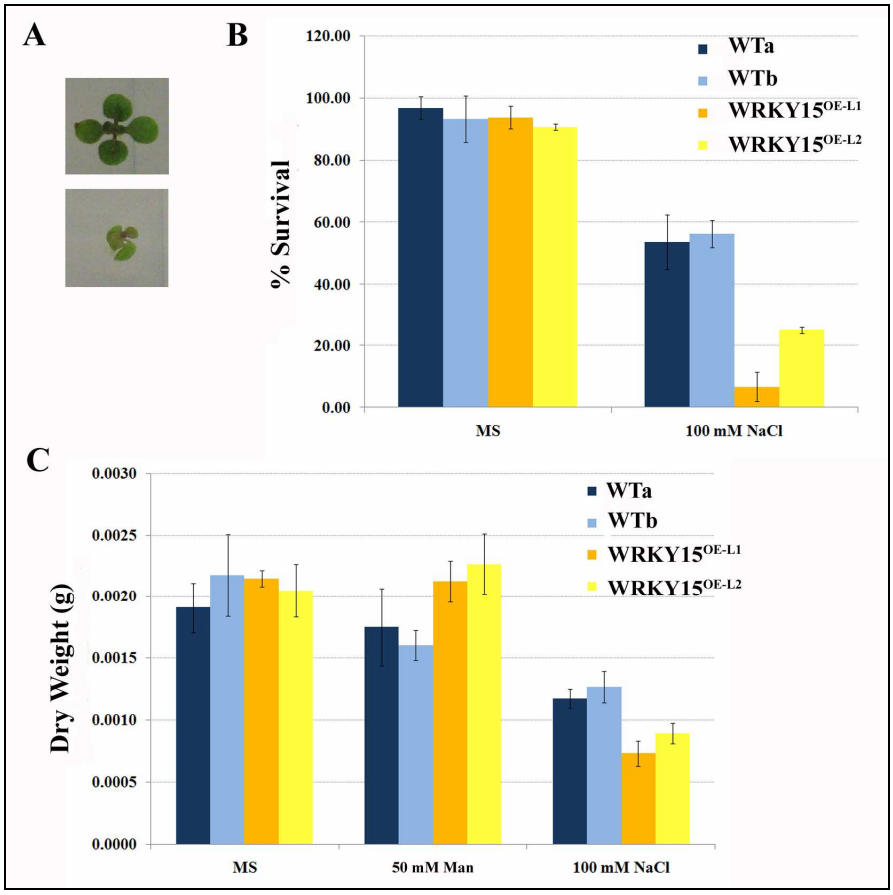

Figure 7

Involvement of WRKY15 in the response of plants to abiotic stress. **A** Phenotype of a surviving plant (top) and non-surviving plant (bottom) on 100 mM NaCl. **B** Survival rate of WRKY15^{OE} and WT plants to 100 mM NaCl (n=3, error bars are standard deviation). **C** Dry weight of 23 days-old plants grown on MS, 50 mM mannitol or 100 mM NaCl (n=3, error bars are standard deviation). WTa and WTb represent two different seed stocks, WRKY15^{OE-L1} and WRKY15^{OE-L2} are independent transformants.

Co-expression analysis of WRKY15

To get more insight in the molecular function of WRKY15, potential targets or upstream regulators of WRKY15 were identified based on co-expression analysis (Atted II; http://www.atted.bio.titech.ac.jp; Obayashi *et al*., 2007). The WRKY15 co-regulated genes were highly enriched for signal transduction (especially TFs, protein kinases), and calcium, and disease responses. Co-expressed TFs include many WRKY TFs, including WRKY6, 11, 22, 25, 33 and 40, as well as several protein kinases that are co-expressed with WRKY15. These protein kinases include CRK11 (AT4G23190), BIK1 (AT2G39660), CPK28 (AT5G66210), and MKS1 (AT3G18690), MPK3 (AT3G45640) and MEKK1 (AT4G08500) (Table 2).

Table 2. WRKY15 coexpressed transcription factors and protein kinases

AGI	Description	Correlation coefficient
Protein kinases		
AT4G23190	CRK11 (CYSTEINE-RICH RLK11)	0.73
AT3G09830	protein kinase, putative	0.71
AT5G25930	leucine-rich repeat family protein / protein kinase family protein	0.71
AT2G39660	BIK1 (BOTRYTIS-INDUCED KINASE1)	0.7
AT3G53810	lectin protein kinase, putative	0.67
AT5G66210	CPK28 (calcium-dependent protein kinase 28)	0.66
AT3G18690	**MKS1 (MAP KINASE SUBSTRATE 1)**	0.66
AT3G57530	CPK32 (calcium-dependent protein kinase 32)	0.65
AT3G45640	ATMPK3 (mitogen-activated protein kinase 3)	0.64
AT3G08720	ATPK19/ATPK2 (A*rabidopsis thaliana* serine/threonine protein kinase 19, *arabidopsis thaliana* serine/threonine protein kinase 2)	0.64
AT1G11050	protein kinase family protein	0.64
AT3G28450	leucine-rich repeat transmembrane protein kinase, putative	0.64
AT2G02220	ATPSKR1 (PHYTOSULFOKIN RECEPTOR 1)	0.63
AT1G01560	ATMPK11 (*Arabidopsis thaliana* MAP kinase 11)	0.62
AT1G14370	APK2A (PROTEIN KINASE 2A)	0.61
AT4G32300	lectin protein kinase family protein	0.61
AT1G16670	protein kinase family protein	0.61
AT2G33580	protein kinase family protein / peptidoglycan-binding LysM domain-containing protein	0.61
AT1G74360	leucine-rich repeat transmembrane protein kinase, putative	0.61
AT1G09970	leucine-rich repeat transmembrane protein kinase, putative	0.61
AT4G08500	**MEKK1 (mitogen activated protein kinase kinase)**	0.61
AT3G46930	protein kinase family protein	0.6
AT5G61560	protein kinase family protein	0.6
AT5G47070	protein kinase, putative	0.6
Transcription factors		
AT2G38470	**WRKY33 (WRKY DNA-binding protein 33)**	0.76
AT5G41100	DNA binding	0.69
AT4G18880	AT-HSFA4A (*Arabidopsis thaliana* heat shock transcription factor A4A)	0.67
AT1G80840	WRKY40 (WRKY DNA-binding protein 40)	0.67
AT1G18570	MYB51 (myb domain protein 51)	0.66
AT5G27420	zinc finger (C3HC4-type RING finger) family protein	0.66
AT1G42990	ATBZIP60 (BASIC REGION/LEUCINE ZIPPER MOTIF 60)	0.65
AT4G17500	ATERF-1 (ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 1)	0.65
AT5G63790	ANAC102 (*Arabidopsis* NAC domain containing protein 102)	0.64
AT1G62300	WRKY6 (WRKY DNA-binding protein 6)	0.64
AT3G55980	zinc finger (CCCH-type) family protein	0.64
AT1G27730	STZ (SALT TOLERANCE ZINC FINGER)	0.63
AT2G40140	CZF1/ZFAR1	0.63
AT4G17490	ATERF6 (ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 6)	0.62
AT4G17230	SCL13 (SCARECROW-LIKE 13)	0.62
AT4G01250	WRKY22 (WRKY DNA-binding protein 22)	0.62
AT3G13430	zinc finger (C3HC4-type RING finger) family protein	0.62
AT2G30250	WRKY25 (WRKY DNA-binding protein 25)	0.61
AT5G59550	zinc finger (C3HC4-type RING finger) family protein	0.61
AT3G49530	ANAC062 (*Arabidopsis* NAC domain containing protein 62)	0.6
AT5G05140	transcription elongation factor-related	0.6
AT4G31550	WRKY11 (WRKY DNA-binding protein 11)	0.6
AT2G42360	zinc finger (C3HC4-type RING finger) family protein	0.6

Genes that are discussed in the text are indicated in bold.

DISCUSSION

H_2O_2 is an important signaling molecule during the stress adaptation of plants by regulating the expression of genes of which the encoded proteins are involved in protection or signal amplification, the latter include TFs and protein kinases (Mittler *et al*., 2004). In recent years, it has become clear that stress-inducible TFs are extremely potent inducers of durable and multiple stress tolerance because they regulate the expression of many defense genes (Fujita *et al*., 2005; Kobayashi *et al*., 2007; Ogawa *et al*., 2007; Tang *et al*., 2007b; Xu *et al*., 2007). In this chapter, we have initiated an effort to analyze the role of H_2O_2 as signal molecule during the plants' adaptation to abiotic stress by evaluating transgenic *Arabidopsis* plants that ectopically express H_2O_2-induced regulatory genes for tolerance against oxidative and heat stress.

Because the expression of the genes that were selected for this study was induced by H_2O_2, we first tested the corresponding transgenic lines for tolerance against oxidative stress. Tolerance to oxidative stress can be evaluated by exogenous application of ROS-producing herbicides, such as paraquat / methyl viologen (superoxide), and rose bengal (singlet oxygen), or alternatively, by using peroxides, such as tert-butyl peroxide and H_2O_2. In this work, plants were exposed to oxidative stress by adding H_2O_2 to the growth medium. High temperatures induce the accumulation of ROS which control the expression of genes that can protect against the detrimental effects of heat stress (Dat *et al*., 1998; Vanderauwera *et al*., 2005). Therefore, the potential of H_2O_2-induced genes to improve heat tolerance of plants was also tested. Two different genetic processes contribute to heat tolerance: Basal thermotolerance, which is the inherent ability to tolerate temperatures above the optimal for growth, and acquired thermotolerance induced by pre-exposure to moderately high temperatures (Larkindale *et al*., 2005). Since the transgenic lines constitutively overexpress the transgenes, it was expected that de potential defense mechanism would already be active and therefore, it was decided to test for basal thermotolerance. Heat stress tolerance of $HSP101^{OE}$ and $DREB2A^{OE}$ transgenic lines could be confirmed, which illustrates the robustness of the assay.

Yield penalty associated with ectopic expression of H_2O_2-induced genes

Ectopic expression of five genes (encoding WRKY33, LRR, ATAF1/ANAC002, ANAC059 and snRNP proteins) resulted in chlorosis and a decrease in rosette size under normal conditions, and thus negatively affected plant growth. Two of these genes, ATAF1/ANAC002 and ANAC059, belong to a large family of plant-specific transcription factors, defined by the presence of a NAC (petunia NAM *Arabidopsis* ATAF1/2, and CUC2) domain, and with widespread functions during different developmental programs, defense against pathogens and abiotic stress (*Arabidopsis* Genome Initiative; http://www.arabidopsis.org; Olsen *et al*., 2005; Wortman *et al*., 2003). ATAF1/ANAC002

itself is highly induced by abscisic acid (ABA), drought, high salinity, osmotic stress and high temperatures, which hints towards an important role during the plants' response to abiotic stresses (Lu *et al*., 2007). Recently, it was shown that ATAF1 negatively regulates the expression of known ABA and stress-responsive genes, including COR47, ERD10, KIN1, RD22 and RD29A, consequently, ataf1 mutants showed enhanced tolerance to drought (Lu *et al*., 2007). Co-expression analysis with Atted-II (http://www.atted.bio.titech.ac.jp; Obayashi *et al*., 2007), combined with gene ontology analysis of the co-regulated genes indeed showed that several of the ATAF1 co-regulated genes, including ABI1 and HAB1, are involved in ABA-signaling. ABA is an important phytohormone in plants that regulates diverse physiological and developmental processes, including senescence, seed germination and stomatal closure (Christmann *et al*., 2006), and constitutive expression of ABA-responsive genes can result in a yield penalty under normal conditions (Haake *et al*., 2002; Kim *et al*., 2004). It is possible that ectopic expression of ATAF1 results in constitutive activation or repression of ABA responses, which would explain the observed yield penalty of $ATAF1^{OE}$ transgenic lines.

Ectopic expression of WRKY15 increases tolerance of plants to oxidative stress

Our results indicated that ectopic expression of WRKY15 increases rosette area of plants. In addition, we showed that WRKY15 positively regulates the tolerance of plants to H_2O_2-induced oxidative stress. WRKY protein comprise a large family of plant-specific TFs that all contain a cognate WRKY DNA-binding site (*Arabidopsis* Genome Initiative; http://www.arabidopsis.org; Wortman *et al*., 2003). WRKY15 belongs to the subfamily IId, which consist of proteins with one WRKY domain, a CCHH zinc finger motif and a calmodulin-binding domain (Eulgem *et al*., 2000; Park *et al*., 2005; Ülker *et al*., 2004). Several IId members, including WRKY7, WRKY11 and WRKY17, are known to act as negative regulators of resistance against pathogens, but no specific role for WRKY15 during disease resistance has been described yet (Eulgem and Somssich, 2007).

The promoter of WRKY15 contains a W-box element (TTTGACC/T), which points to autoregulation or regulation by other WRKY TFs. Of all WRKY15 co-expressed genes, the correlation coefficient of WRKY33 was the highest, making it the most likely candidate for regulation of WRKY15 expression. Expression of WRKY33 is also rapidly induced by pathogens or pathogen-mimicking molecules, and it is known that WRKY33 acts downstream of PAD4 (phytoalexin-dependent 4), a key regulator upstream of salicylic acid (SA), but upstream of SA to regulate the expression of SA-dependent defense genes (Lippok *et al*., 2007). Co-expression analysis of WRKY15 showed that its expression is correlated not only correlated with that of WRKY33, but also with that of MKS1 (MAP kinase substrate 1) and MEKK1. WRKY33, MKS1 and MEKK1 all function in the same

signaling pathway (Figure 8). The expression of WRKY33 is partly under negative control (Lippok *et al*., 2007), which might be indirectly mediated by the AP2C1 phosphatase through inhibition of MPK4 (mitogen-activated protein kinase 4) (Schweighofer *et al*., 2007). MPK4 probably regulates WRKY33 activity via interaction with MKS1 (Andreasson *et al*., 2005).

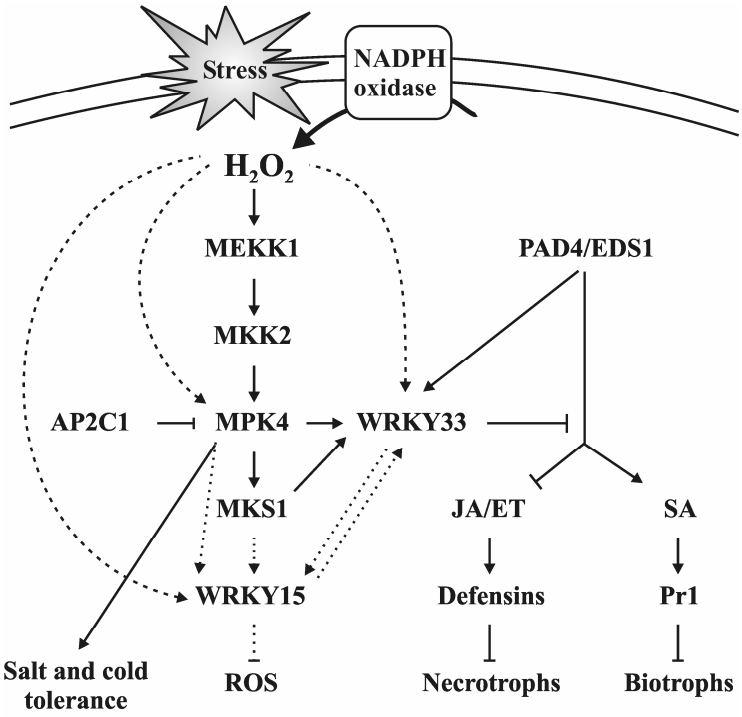

Figure 8

Hypothetical model for WRKY15 action based on co-expression with genes of which the products have known functions. Central points in the model include: WRKY15 is induced by H_2O_2 (dashed lines) and protects plants from oxidative stress. WRKY15 expression is highly correlated with that of WRKY33. WRKY33 is a target of MPK4, which functions in an ROS-activated MAPK module. MPK4 negatively regulates both activities of EDS1 and PAD4. EDS1 and PAD4 act as activators of SA- induced defense gene expression (systemic acquired resistance) and repressors of the ET/JA pathway. Full lines represent known interactions (see text). Dotted lines indicate hypothetical interactions.

The MPK4-MKS1-WRKY33 pathway has known antagonistic functions during disease resistance: It is required for repression of salicylic acid (SA)-dependent systemic acquired resistance and for activation of jasmonate (JA)- and ethylene (ET)-dependent defense gene expression (Andreasson *et al*., 2005; Broderson *et al*., 2006; Peterson *et al*., 2000; Zheng *et al*., 2006). Induction of plant defensins by WRKY33 via the JA/ET pathway is required for resistance against necrotrophic pathogens, while repression of PR1 (pathogenesis related 1) by WRKY33 enhances susceptibility to *Pseudomonas syringae* (Andreasson *et al*., 2005; Zheng *et al*., 2006).

The expression of MPK4 and WRKY33 was induced by H_2O_2 in catalase-deficient plants, showing that the pathway is at least partly controlled by H_2O_2 at the transcriptional level. (Figure 1). It is noteworthy that MPK4 can be activated by MEKK1 in a ROS-controlled manner and that this mechanisms regulates the expression of genes involved in redox control (Nakagami *et al*., 2006). It has been shown that MPK4 can be activated by MKK2 (MAP kinase kinase 2) to mediate cold and salt responses, and expression of WRKY33 is highly induced by cold, salt and osmotic stress, suggesting that MPK4 and WRKY33 function at the cross-road of both abiotic and biotic stress responses (Brader *et al*., 2007; Teige *et al*., 2004). Our data showing that overexpression of the H_2O_2-induced WRKY15 gene increases resistance to oxidative and osmotic stress, but reduces tolerance to salt stress, further support a role for WRKY15 in H_2O_2-signal transduction during the response of plants to abiotic stress.

MPK4^{OE} plants did not show obvious differences with WT plants under oxidative stress, but the screen indicated that these plants were more tolerant to heat stress. WRKY33^{OE} plants showed extensive chlorosis under control conditions suggesting that WRKY33 regulates additional processes that are necessary for normal plant physiology (Figure 9).

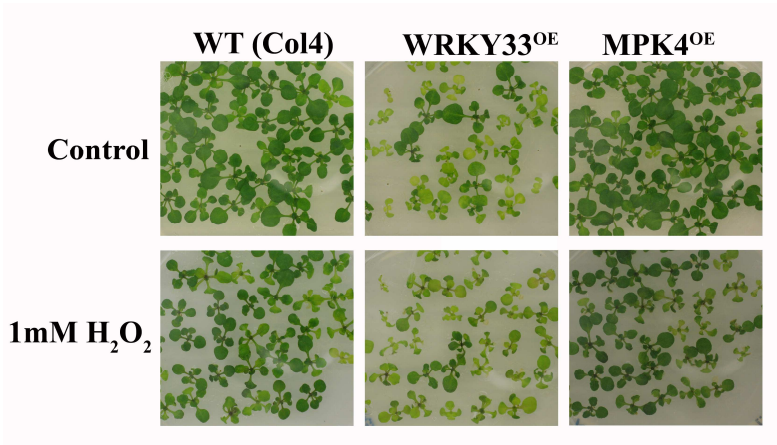

Figure 9

Phenotype of WRKY33^{OE} and MPK4^{OE} transgenic plants. Plants were grown under control and oxidative stress (1.0 mM H_2O_2) conditions.

CONCLUSIONS AND PERSPECTIVES

Transgenic *Arabidopsis* plants with increased levels of H_2O_2-inducible genes were screened for altered tolerance to oxidative and heat stress. Several transgenic lines, including lines that constitutively express ATAF1, were stunted and showed bleaching under control conditions. This indicates that ATAF1 negatively regulates the growth and development of plants.

Our data indicated that ectopic expression of the WRKY15 TF increases rosette size and improves tolerance to increased levels of H_2O_2. Furthermore, WRKY15^{OE} plants were more tolerant to osmotic stress and more sensitive to salt stress. Taken together, our data suggest that WRKY15 is an important component of H_2O_2 signal transduction and plays an important role during the abiotic stress response of plants. Since the plant response to stress is coordinated at the transcriptional level, it would be highly relevant to perform a molecular profiling of the WRKY15 transgenic. An pilot cDNA-AFLP analysis indicated that the expression of (at least) two genes, encoding a major intrinsic family protein (AT2G36830) and a nitrilase 4 (AT5G22300), is controlled by WRKY15. A Gabi-kat T-DNA insertion line in the promoter of the WRKY15 gene is being sorted out so that we also can study the phenotype of loss-of-function mutants. This work is being followed-up in order to publish the data in a high impact factor journal.

MATERIALS AND METHODS

Production of transgenic *Arabidopsis thaliana*, overexpressing H_2O_2-responsive signal transduction components

The production of the transgenic lines that were used in this work is described by Vanderauwera (2007). In short, full-length cDNAs were PCR-amplified with *Pfu* proofreading DNA polymerase (Promega, Madison, USA) by using gene-specific primers flanked by partial att*B* sites, reamplified using full length *att*B1 and *att*B2 primers, and cloned into the Gateway entry vector pDONR221 (Invitrogen, Carlsbad, CA) and the binary destination vector pB7WG2D (Karimi *et al*., 2002). Constructs were transformed into *Arabidopsis thaliana* Col-4 wild type plants through *Agrobacterium*-mediated floral dip transformation (Clough and Bent 1998), primary transformants were selected through resistance to kanamycin and allowed to self-fertilize. Two independent homozygous overexpression lines (using Northern) with single T-DNA insertions (using segregation analysis) were selected for further analysis.

Plant material and growth conditions

All experiments were performed with *Arabidopsis* seeds from wild type Col-4 and homozygous transgenic plants that were grown on the same tray under controlled growth conditions. For *in vitro* experiments, *Arabidopsis* seeds were sterilized by incubation with 5% NaOCl and grown on 4.3 g/l Murashige-Skoog (MS; Duchefa, Haarlem, The Netherlands), 0.5 g/l MES, 0.1 g/l myo-inositol, 10 g/l sucrose, 9 g/l plant tissue culture agar (LabM, Bury, UK), 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxin and 1 mg/l thiamin at 22°C and 65 µE m^{-2} s^{-1} radiation under a continuous light regime (heat, cold and oxidative stress), or under a 16/8 h light/dark cycle (salt and osmotic stress) assays. Seeds were stratified by incubation at 4 °C in the dark. Screening for stress tolerance was performed with seeds of WT and transgenic plants that were grown in the greenhouse during the summer of 2006. For the screening, 40 seeds were placed per growth plate (Ø 8.5 cm) containing 40 ml medium. Confirmation experiments with WRKY15 and ATAF1 transgenic lines were performed with seed batches that were harvested from plants grown during spring 2007. For these experiments, 30 seeds per genotype were grown in quadrants on the same growth plate (Ø 14 cm) containing 100 ml medium.

Stress assays

Oxidative stress experiments were performed by germinating and growing seeds on plates containing various concentrations of H_2O_2 (0-10 mM H_2O_2). All screening experiments were done on 0 and 1 mM, while 0, 0.5, 1.0 and 1.5 mM H_2O_2 was used for confirmation experiments. For heat stress, one week-old plants were placed in a thermostat cabinet (Lovibond) at 38 °C for 12 h. Plants were recorded via digital imaging after one and two weeks (oxidative stress) or after 0, 1, 2, 3 and 7 days recovery from the heat shock (heat stress).

Salt and osmotic stress assays were performed by germinating and growing seeds on plates containing NaCl or mannitol, respectively. Cold stress treatments were done by germinating and growing seeds on MS plates at 12 °C.

Leaf series or dry weight analysis were done 23 days after transfer to the growth chamber. Digital images of leaf series were analyzed using ImageJ software.

Quantification of rosette area

Digital images were used for quantification of total rosette area. Images were background-corrected with an in house developed image analysis software (based on the SDC Morphology Toolbox for MATLAB; http://www.mmorph.com). First, the program performs a RGB split and the blue color was retained to obtain a better contrast between plants and background.

An arbitrary threshold was set to separate the plants from the background, the obtained images were then corrected for residual noise (based on the amount of joined pixels).

Finally the total rosette area of all plants from the same genotype grown on one growth plate was calculated and these values were manually corrected for the number of germinated plants on the plate.

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PART III

DROUGHT STRESS IN *ARABIDOPSIS*

CHAPTER 5

Development and evaluation of a semi-automated system to monitor the growth of *Arabidopsis* plants during drought stress

ABSTRACT

Environmental stresses, including drought, have a negative impact on plant growth and yield. In order to decipher the molecular networks regulating growth responses under drought stress conditions, we compared the growth of stress-tolerant plants during water-limited conditions. A semi-automated platform that allowed controlling soil water concentrations was designed. This platform, which we called WIWAM (weighing, imaging and watering machine), was used to monitor the growth of wild type (WT) *Arabidopsis* plants, as well as transgenic plants that ectopically express AVP1 (encoding a vacuolar H^+-pyrophosphatase) and GOLS2 (encoding an enzyme involved in galactinol biosynthesis). It was published that $AVP1^{OE}$ plants have an increased rosette size compared to WT plants and that these plants are more tolerant to drought and salt stress, while $GOLS2^{OE}$ plants were shown to be more tolerant to drought stress. Our data showed that the increased rosette size of $AVP1^{OE}$ plants under normal conditions is due to an increased growth rate. In contrast, growth rate and rosette size of GOLS2 was reduced compared to WT plants under both control and drought conditions. We conclude that WIWAM allows to analyze the growth of soil-grown plants under controlled watering conditions and hence offers a platform to analyze the response of plants to drought stress.

INTRODUCTION

Plants are vital for the welfare of humans as food, feed and bio-energy sources, but their yield and quality is directly affected by climate and weather (Porter and Semenov, 2005). Environmental changes caused by global warming have confronted humans with major agricultural problems, especially in developing countries that fully depend on the cultivation of crop species. Moreover, the rapidly rising world population has led to a proportional increase in food and feed demand that needs to be dealt with. Therefore, improving crop yield, both under normal and unfavorable conditions, will be essential for securing a sustainable future.

Among all environmental stresses, drought (which is defined as an unfavorable period of below normal water precipitation) is one of the greatest global constraints for agriculture (Boyer, 1982). Climate changes caused by global warming are expected to engrave drought-related problems by limiting water access (Blashky *et al*., 2007). Drought stress can also be associated with water shortage caused by altered soil salinity or ice formation at freezing temperatures (Levitt, 1980; Zhu, 2001a). Common to all drought conditions is that they limit water availability (quantified as decrease in water potential, Ψ_W), which ultimately lead to dehydration of plant cells. Plants must adapt to cellular dehydration in order to survive and this adaptation response can best be explained by the avoidance/tolerance theory, which is also called the homeostasis/protection model (Levitt, 1972; Zhu, 2001b). An excellent description of this theory is provided by Verslues and coworkers (2006). Initially, plants try to avoid a decrease in cellular water potential and maintain homeostasis by improving water uptake through an increased root/shoot ratio, as well as increasing water storage and decreasing water loss via increased cuticle thickness and abscisic acid (ABA)-induced stomatal closure. A second response of plants involves lowering of the cellular Ψ_W to circumvent water loss from the cells, which occurs when then soil Ψ_W is lower than the cellular Ψ_W. Such a response is called dehydration avoidance and occurs through the accumulation of compatible solutes (osmotic adjustment). However, when the stress becomes even more severe, avoidance of low Ψ_W and dehydration will be insufficient to maintain cellular homeostasis. Then, mechanisms to tolerate dehydration will become more important and these include the protection of cellular structures, for example by increased synthesis of late-embryogenesis-abundant (LEA) proteins. Also the level of reactive oxygen species and the damage that they cause need to be controlled (Vinocur and Altman, 2005, see Chapter 1). Although the adaptation of plants to decreased Ψ_W can be summarized into the above explained avoidance/tolerance or homeostasis/protection model, many of the molecular events that are initiated by drought do not fit exclusively into one of the categories within this model and moreover, they do not occur separately in a linear progression of time (Verslues *et al.*, 2006).

Numerous *Arabidopsis* genes have been described that, when mutated or ectopically expressed, increased tolerance to less favorable conditions (see Chapter 1; Supplementary Table S1). The products of such stress tolerance genes (STGs) are involved in many different biological processes, including various signaling pathways (involving hormones, calcium, protein phosphorylation and transcription factors), RNA processing, protein stability, and ion transport (Yamaguchi-Shinozaki and Shinozaki, 2006; see Chapter 1).

This work is an initial step in a project of which the long-term objectives are to better understand the molecular mechanisms that control plant growth under both optimal and drought stress conditions. A flow-chart describing the followed strategy is presented in Figure 1. I contributed to the transgene selection, initial characterization of the transgenic lines and the establishment of the drought assays. Here, the objective was to analyze the growth of transgenic *Arabidopsis* plants with modified levels of STGs under low water availability. To this end, a semi-automated system was designed that allowed stabilizing and controlling the water concentration in individual soil-grown plants.

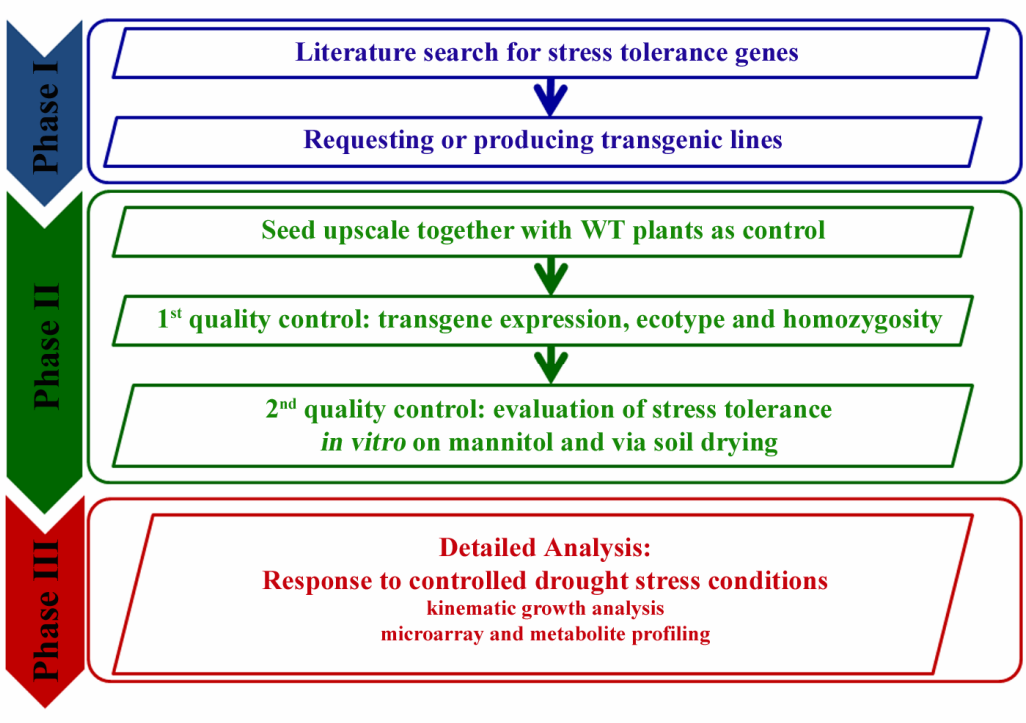

Figure 1

Flow chart showing the followed strategy in this study. Phase I represents literature data mining to identify the stress tolerance genes, and requesting or producing the corresponding transgenic lines. Phase II consists of seed bulking and two series of quality controls. Phase III is a detailed functional analysis of transgenic lines.

RESULTS

Selection and quality control of *Arabidopsis* lines with enhanced stress tolerance

A first necessary step in order to analyze the growth of stress-tolerant *Arabidopsis* plants with modified levels of STGs under limited water conditions was to collect such transgenic lines. Although the focus was on drought stress, we also included transgenic lines with increased tolerance to other abiotic stresses than drought, such as salt, heat, cold, freezing and oxidative stress, because STGs can be involved in cross-tolerance to multiple stresses. To avoid additional levels of complexity, heterologous genes were excluded. An extensive literature search using PubMed or Web of Science yielded approximately 150 *Arabidopsis* STGs (see Chapter 1: Supplementary Table S1). For practical reasons, this STGs list was refined based on different criteria, and these include the genetic background in which they were transformed (Columbia*,* Col), the absence of reported negative growth effects of the transgenic plants and the quality of the paper, which resulted in a selection of approximately 40 STGs.

STGs, 18 in total, for which the corresponding transgenic lines are currently present in the lab are listed in Table 1. The received transgenic seeds have been bulked together with WT (Col) to avoid seed effects that could influence the read-out of the stress experiments. Quality control of the received transgenic lines was performed and included ecotype confirmation via satellite fingerprinting of genomic DNA, segregation analysis to test for homozygosity and transgene expression. The transgenic lines were also screened for drought stress tolerance *in vitro* using mannitol and by soil-drying (see Supplementary Data, provided at the end of this chapter, for results of the quality controls that were performed on the transgenic lines). The final step in this part of the project is to analyze the growth of transgenic lines during low water availability.

Table 1. Overview of received transgenic lines

Gene	AGI	Description	Construct	Stress	Reference
Transcription					
ICE1	AT3G26744	transcription factor	OE (Supermas)	C, F	Chinnusamy *et al*., 2003
MBF1c	AT3G24500	transcriptional co-activator	OE (35S)	H, O	Suzuki *et al*., 2005
MYB60	AT1G08810	transcription factor	tDNA KO	D	Cominelli *et al.*, 2005
Signal Transduction					
CaMBP25	AT2G41010	calmodulin-binding protein	AS (35S)	O, S	Perruc *et al*., 2004
SRK2C	AT1G78290	SNF1-related protein kinase 2	OE (35S)	D	Umezawa *et al*., 2004
Osmoprotection					
GOLS2	AT1G56600	galactinol synthase	OE (35S)	D	Taji *et al*., 2002
TPS1	AT1G78580	trehalose-6-P synthase	OE (35S)	D	Avonce *et al*., 2004
Ion homeostasis					
AVP1	AT1G15690	vacuolar H(+)-pyrophosphatase	OE (35S)	D, S	Gaxiola *et al*., 2001
NHX1	AT5G27150	Na+/H+ antiporter	OE (Supermas)	S	Apse *et al*., 1999
SOS1	AT2G01980	plasma membrane Na+/H+ antiporter	OE (35S)	S	Shi *et al*., 2003
Redox/energy homeostasis					
tAPX	AT1G77490	ascorbate peroxidase	OE (35S)	Ox	Murgia *et al*., 2004
AOX1a	AT3G22370	alternative oxidase	OE (35S)	C	Fiorani *et al*., 2005
RCI3	AT1G05260	cell wall peroxidase	OE (35S)	D, S	Llorente *et al*., 2002
Protein folding/stability					
HSP101	AT1G74310	heat shock protein	OE (35S)	H	Queitsch *et al*., 2000
HSP17.6a	AT5G12030	heat shock protein	OE (35S)	S	Sun *et al*., 2001
Hormone signaling					
NCED3	AT3G14440	9-cis-epoxycarotenoid dioxygenase (ABA biosynthesis)	OE (35S)	D	Iuchi *et al*., 2002
RNA stability/metabolism					
RZ-1a	AT3g26420	glycine rich RNA binding protein	OE (35S)	C, F	Kim *et al*., 2005
SRL1	AT5g37370	splicing factor	OE (35S)	S	Forment *et al*., 2002

The promoter to generate the construct is shown between brackets. 35S, cauliflower mosaic virus constitutive 35S promoter. OE, overexpression; AS, antisense; KO, knock-out; C, cold; F, freezing; D, drought; H, heat; O, osmotic; Ox, oxidative; S, salt stress.

Development of a semi-automatic system for growth analysis during controlled drought stress

To monitor plant growth during controlled watering conditions in soil, we first developed a (semi-) automatic system, similar to the PHENOPSIS platform, that allowed controlling and stabilizing the soil water status in transpiring plants (Granier *et al*., 2006). Basically, this system, which we called WIWAM (weighing, imaging and watering machine), consists of a barcode reader, digital camera, scales and pump, which are all connected to a computer (Figure 2*A*). One run per individual sample includes (in chronological order): recording of the sample identity, weighing of the sample (pot, soil and plant), imaging of the plant rosette, watering of the plant and saving the data on the computer. This run was completed on a daily basis so that plants were watered each time with the amount necessary to reach a given soil water concentration (SWC), which was defined based on the retention capacity of saturated soil and used to calculate a total target weight (TW_{Total}) for each sample (see Materials and Methods for a detailed description). TW_{Total} of each

sample is saved in an EXCEL (Microsoft) file on the computer. After identification of the sample with the barcode reader, sample weight is measured with the scales and this is compared by the computer with the TW_{Total}. The computer then activates the pump to add water until the TW_{Total} is reached. The daily water delivery was managed independently for each pot so plants with higher transpiration rates received more water than those with lower transpiration rates. Since the weight of the plant itself is negligible (< 5 %), the only significant variable in the measured total weight of each sample is the amount of water. Thus, by daily compensating for the loss of water, the amount of available water for each plant could be stabilized. Each day, a digital image of the plant rosette is made with the camera (which is placed above the plant) and this can be used for quantification of the rosette area (see Materials and Methods for detailed description). The output per run and per sample is a digital picture with the name including sample identity and sample weight before watering. This protocol could be performed manually on a small scale but has been automated here, allowing a throughput of maximum 100 plants per hour.

Figure 2*B* shows the change in total sample weight for a preliminary experiment in which plants were exposed to different SWCs by setting three different total target weights (TW_{tot}). TW1 was calculated based on a SWC of 2.0 g H_2O / g dry soil, TW2 was calculated based on a SWC of 1.5 g H_2O / g dry soil, and TW3 was calculated based on a SWC of 0.0 g H_2O / g dry soil and thus equaled the sum of dry soil and empty pot. Sample weight of plants with TW1 or TW2 was kept constant and these plants were thus exposed to stable amounts of water. As TW3 samples never received water, they were used as negative control and their total weight declined gradually until the SWC was 0.0 g H_2O / g dry soil. We conclude that this system is useful for controlling and stabilizing the soil water status during drought stress experiments.

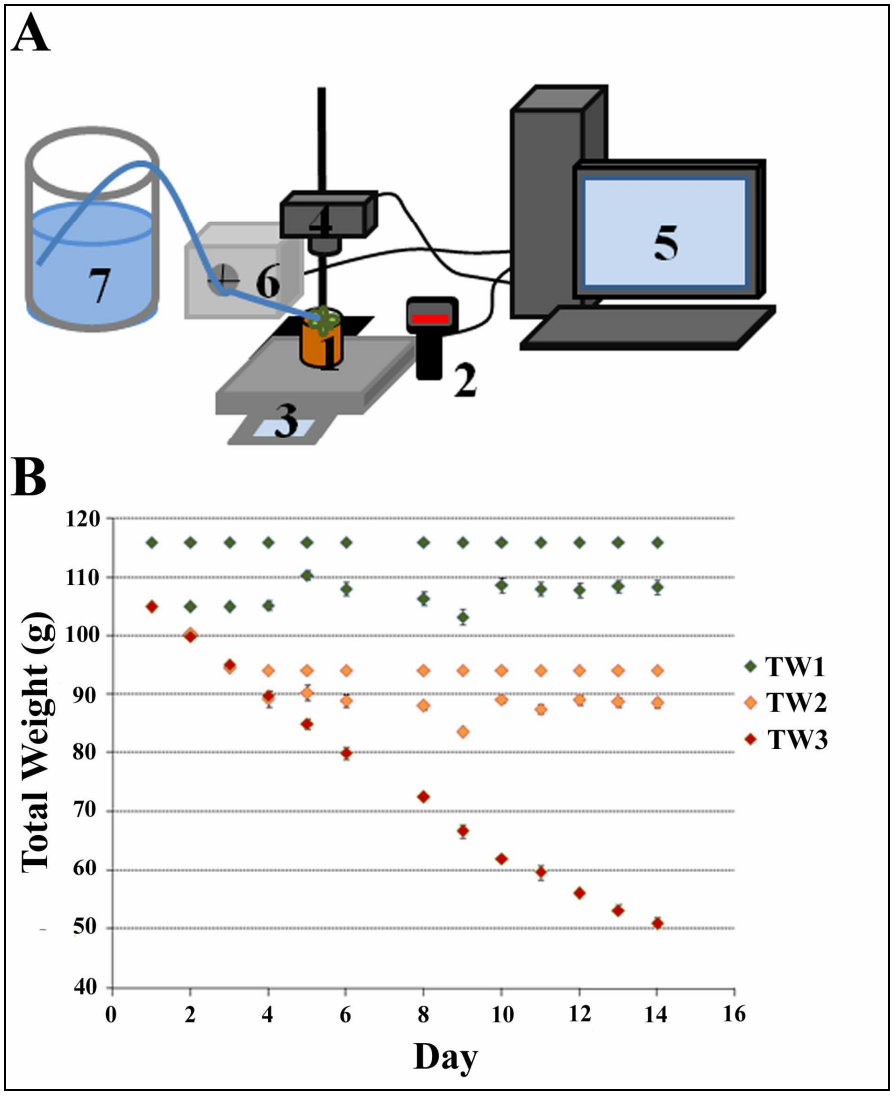

Figure 2

A Design of WIWAM, a semi-automatic system for imposing and monitoring drought stress during plant growth: plant sample (1), barcode scanner (2), scales (3), camera (4), computer (5), pump (6), water reservoir (7). **B** Time curve showing the daily change in total weight, as measured with WIWAM for two weeks, before and after rewatering of samples with different target weight (TW) values (n=10, errors bars represent standard error of the mean). Green and orange represent controlled rewatering treatment of plants to maintain TW, while red represents no watering.

Analysis of WT plants under controlled drought conditions

We first performed a pilot experiment with WIWAM to test the effect of drought stress conditions on the growth of *Arabidopsis* Col-0 (WT) plants. WT plants (stage 1.04, Boyes *et al.*, 2001) were subjected to five different drought stress treatments (2.00, 1.75, 1.50, 1.25 and 1.00 g H_2O / g dry soil).

Twenty WT *Arabidopsis* plants were first grown under control watering (1.75 g H_2O / g dry soil) for two weeks. These plants were divided into five equal groups, subjected to the different treatments while rosette development was recorded until completion of rosette stage (Figure 3). This took 10-12 days in all experiments that were performed on *Arabidopsis* Col-0 plants so far. Different parameters, including rosette area, plant biomass and leaf number were scored. Plants that were exposed to the same treatment were homogenous (note the low standard deviations) (Figure 4). Dry weight and final rosette size analysis showed that 1.75 g H_2O / g dry soil was the most optimal water concentration, while higher or lower SWCs reduced dry weight and final rosette size (Figure 4*A* and 4*C*). Numbers of rosette leaves was only affected in plants that received the lowest water concentration (1.00 g H_2O / g dry soil), with a average reduction of two leaves (Figure 4*B*).

In summary, our analysis of WT plants under controlled watering conditions with WIWAM showed that WIWAM is a good system to study the effects of drought stress on the growth and development of soil-grown plants.

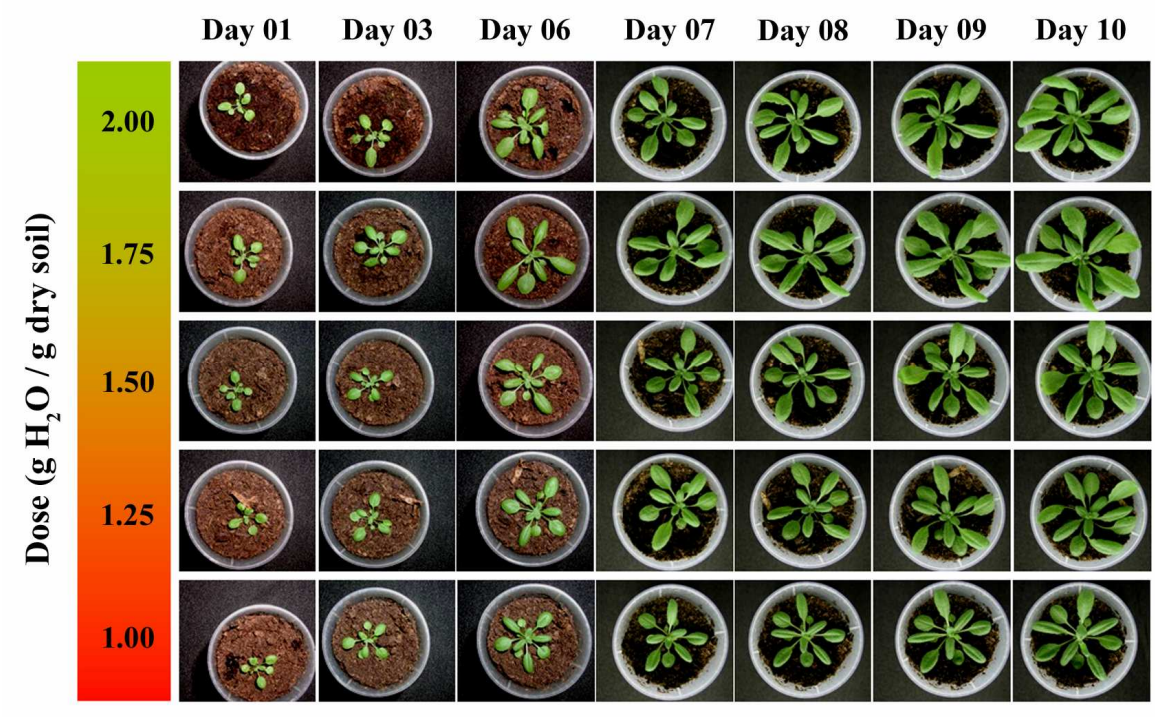

Figure 3

WT plants exposed to five different drought stress doses. Plants were watered and photographed on a daily basis until completion of rosette stage (day10). For each stress dose, one representative plant is shown.

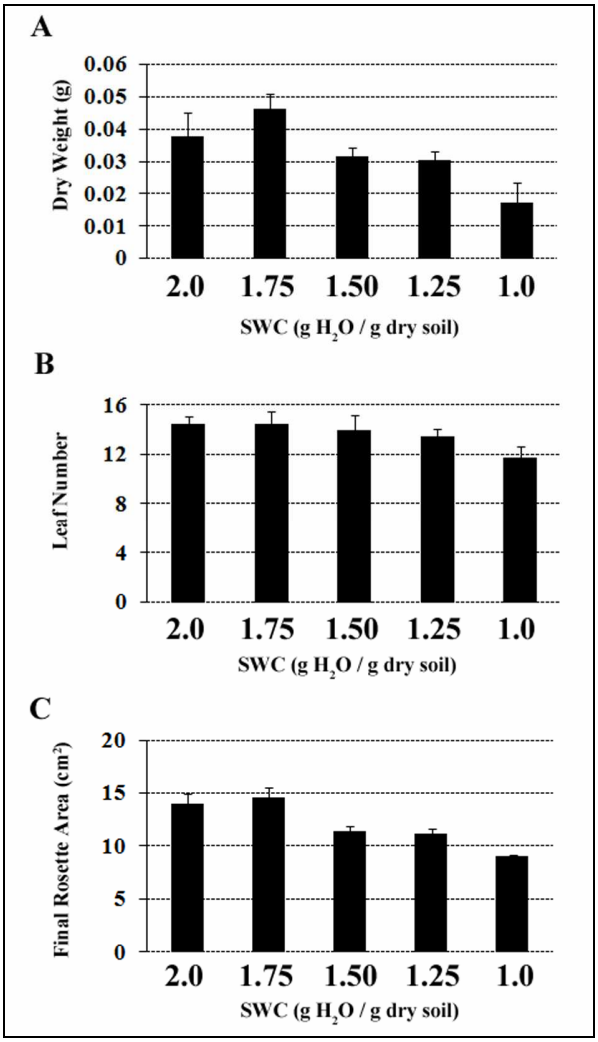

Figure 4

Analysis of the growth response of WT *Arabidopsis* plants exposed to five different drought stress doses. **A** Quantification of dry weight. **B** Quantification of leaf number. **C** Quantification of final rosette area. (n=4, error bars represent standard deviation). SWC, soil water concentration.

WIWAM analysis of AVP1^{OE}and GOLS2^{OE} plants revealed differences in growth rates

The growth of transgenic lines overexpressing AVP1 (AVP1^{OE}) and GOLS2 (GOLS2^{OE}) was analyzed in two preliminary experiments to validate the robustness of the WIWAM system. It was known that the rosette size of AVP1^{OE} plants is increased, and these plants were also shown to be more tolerant to salt and drought stress (Figure 5*A*,*B*; Gaxiola *et al*., 2001; Nathalie Gonzalez, unpublished results). GOLS2^{OE} plants were more tolerant to drought stress (Figure 5*C*; Taji *et al*., 2002). Drought tolerance of GOLS2^{OE} plants was first confirmed by a soil-drying experiment in which 3.5 week-old plants were withheld from watering for two weeks, rewatered and allowed to recover for one day (see Materials and Methods for more details).

Almost all GOLS2^{OE} plants survived the treatment, while half of the WT plants were wilted and could not survive after rewatering (Figure 5*D*).

To compare the growth of the transgenic lines with that of WT during limited water availability, plants were subjected to two different watering regimes, control (1.75 g H_2O / g dry soil) and mild drought stress (1.50 g H_2O / g dry soil). During the treatment, rosette size of each plant was followed and the growth rate was calculated as an increase in rosette size per day. The growth rate of AVP1^{OE} plants was increased under normal conditions compared to WT plants, which resulted in an increased rosette size (Figure 6*A*). However, the growth rate AVP1^{OE} plants was comparable to that of WT plants during the drought treatment. In contrast to AVP1^{OE} plants, GOLS2^{OE} plants showed a decreased growth rate compared to WT plants, resulting in a decreased rosette size (Figure 6*B*).

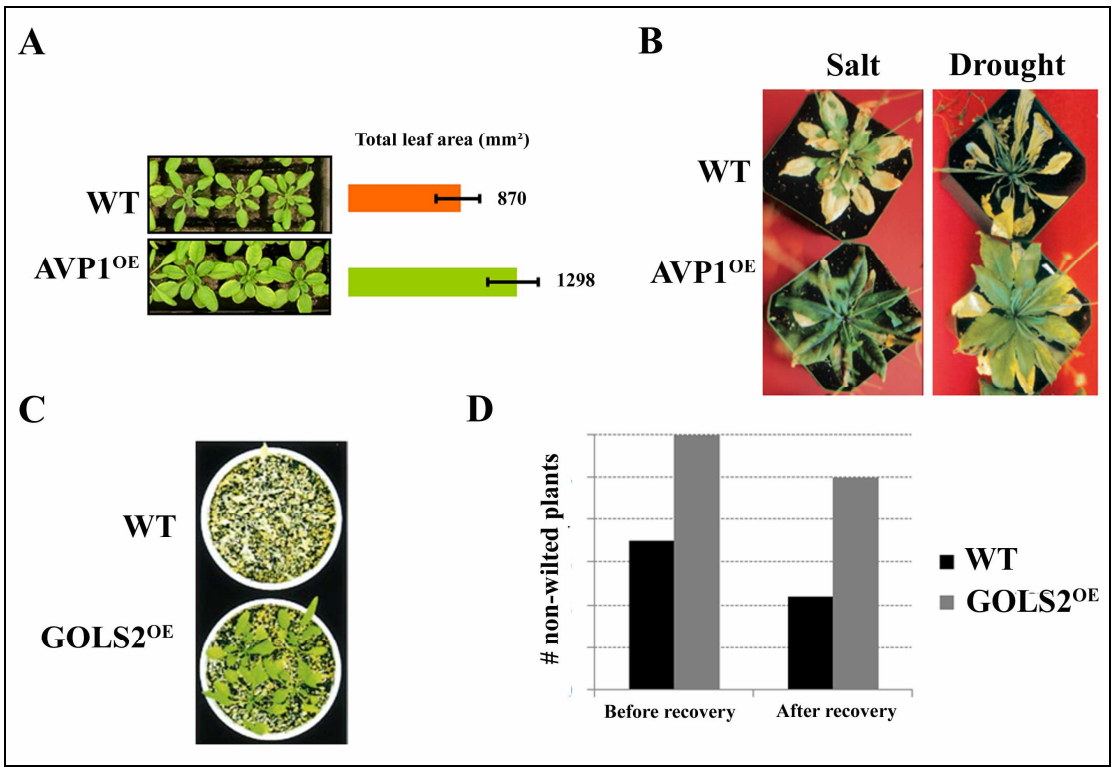

Figure 5

Phenotypes of AVP1^{OE} and GOLS2^{OE} transgenic plants. **A** Enhanced rosette area (total leaf area) of AVP1^{OE} plants (Nathalie Gonzalez, unpublished results). **B** Phenotype of four week-old plants AVP1^{OE} and WT plants exposed to salt or drought stress (adapted from Gaxiola *et al*., 2001). Salt stress was performed by watering with 250 mM NaCl for ten days. Drought stress was performed by withholding water for ten days and rehydration for one day. **C** Phenotype of three week-old plants GOLS2^{OE} and WT plants exposed to stress by withholding water for 14 days and rehydration for five days (adapted from Taji *et al*., 2002). **D** Confirmation of drought tolerance of plants overexpressing GOLS2 by soil drying. Six GOLS2 transgenic and WT plants were grown for 3.5 weeks and withheld from watering for two weeks. Wilting was scored before and one day after rewatering.

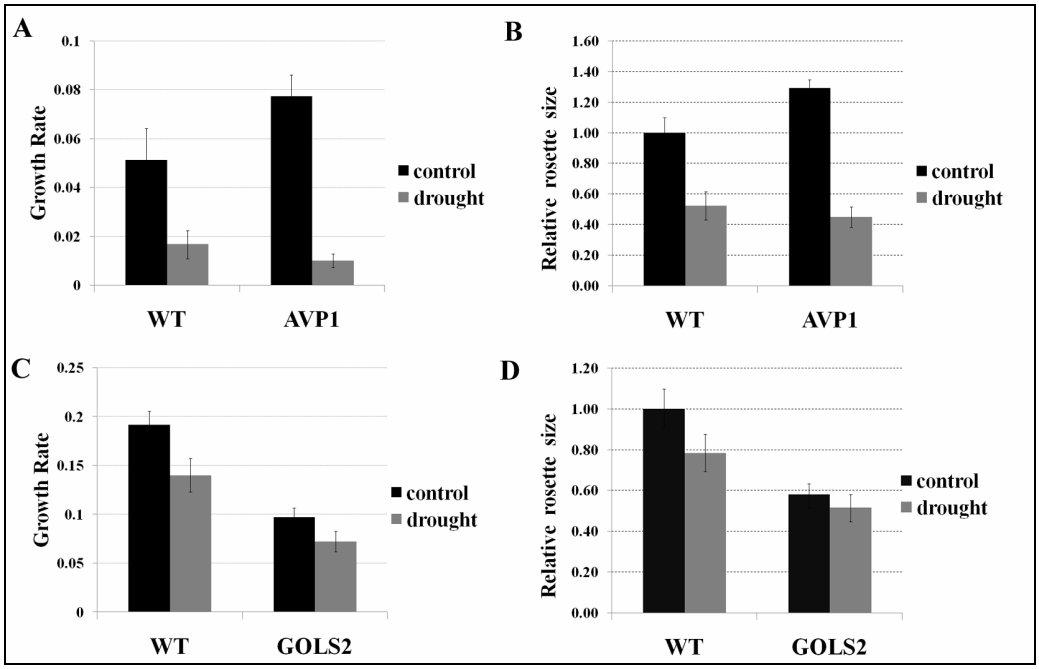

Figure 6

Growth analysis of AVP1^{OE} plants and GOLS2^{OE} plants during controlled watering. Growth rate was determined as the increase in rosette size each day during a period of ten (GOLS2) or 12 (AVP1) days. **A** Growth rate of AVP1^{OE} plants compared to WT plants. **B** Relative rosette size of AVP1^{OE} plants compared to WT plants. **C** Growth rate of GOLS2^{OE} plants compared to WT plants. **D** Relative rosette size of GOLS2^{OE} plants compared to WT plants.

DISCUSSION

Until now, studies on drought stress responses were mainly focused on identifying gene products that are involved in drought tolerance, but the mechanisms that control growth during drought stress are poorly understood. The response of plants to drought stress can be analyzed by measuring water loss of detached plants or by using osmotica to lower the water potential in the medium, but although these approaches are quite straightforward and allow to test many lines within a reasonable short period of time, several drawbacks are described (Verslues *et al*., 2006). The main limitation is that these approaches are not suitable for longer-term experiments due to secondary effects caused by plant detachment or phytotoxicity of the osmoticum. Soil drying provides a good alternative and reflects more the natural situation in the field. However, quantification of the stress effects during soil drying is problematic due to variations in the degree of the stress between different plants within the same treatment. Such variations can be caused by genetic variability (e.g. variations in water use efficiency and water depletion rate) or experimental factors (e.g. differences in initial soil Ψ_W and position in the growth chamber). These difficulties need to be overcome in order to study the mechanisms that control plant growth during stress.

To this end, a semi-automated system (WIWAM), similar as PHENOPSIS (Granier *et al*., 2006) was set-up that allows analyzing the growth of plant during controlled and stabilized watering conditions. Measuring soil Ψ_W is laborious and not easy to perform in small pots, but with the WIWAM system, the problems with measuring Ψ_W are circumvented by defining drought conditions as a certain soil water concentration (amount of water per dry soil). WIWAM was optimized using WT *Arabidopsis* plants and we showed, as expected, that drought negatively affected the growth of plants. Despite differences in experimental conditions between the PHENOPSIS and WIWAM platform, including growth chamber conditions (day length, humidity, light), soil type (1:1 mixture of soil and organic compost versus soil without organic compost for WIWAM) and timing of stress imposition (stage 1.06 for PHENOPSIS versus stage 1.04 for WIWAM), the phenotypic effects of our drought conditions on *Arabidopsis* Col-0 plants was comparable to those observed by Granier and coworkers (2006) using PHENOPSIS (data not shown). WIWAM is semi-automated allowing a throughput of 100 plants per hour, which is still less than the 300 plant per hour in the PHENOPSIS system. The process of making the picture, data transfer to the computer and pumping of the water should be accelerated to increase the throughput of WIWAM.

The main advantage of WIWAM (and PHENOPSIS) is that it allows to monitor plant growth under controlled watering conditions. Here, WIWAM was used to evaluate the growth of $AVP1^{OE}$ plants, which were reported to have an increased rosette size, as well as increased tolerance to salt and drought stress conditions (Gaxiola *et al*., 2001, Li *et al*., 2005; Nathalie Gonzalez, unpublished results). Growth analysis of $AVP1^{OE}$ plants under control watering conditions using WIWAM indicated that the increase in rosette size was due to an increased growth rate. However, when grown under (mild) drought conditions, the growth rate of $AVP1^{OE}$ plants was comparable to that of WT plants. Moreover, $AVP1^{OE}$ plants did not survive better after long term soil drying and we can conclude that $AVP1^{OE}$ plants are not more tolerant to drought stress in our conditions. The growth and stress tolerance phenotypes of $AVP1^{OE}$ plants is associated with increased accumulation of sodium and potassium (Gaxiola *et al*., 2001). It is likely that, in order to maintain its phenotypes, $AVP1^{OE}$ plants needs a large amount of nutrient resources and when these become limiting, for example due to reduced water availability, $AVP1^{OE}$ plants might loose their advantage. Within this context, it is noteworthy that the phenotypes reported by Gaxiola and coworkers (2001) were observed by watering the plants with a nutrient solution. Nevertheless, our data show that, by using WIWAM, we were able to reproduce the reported enhanced rosette size phenotype of $AVP1^{OE}$ plants under controlled watering conditions.

$GOLS2^{OE}$ plants are more tolerant to drought stress due to increased production of raffinose family oligosaccharydes (RFOs), including galactinol and raffinose, which act as osmoprotective

compounds. Enhanced drought tolerance of GOLS2^{OE} plants is also due to a reduced transpiration rate, probably caused by increased stomatal closure caused by RFO-stimulated ABA biosynthesis (Taji *et al*., 2002). We confirmed the increased drought tolerance of GOLS2^{OE} plants by using a straightforward soil-drying experiment. Growth analysis with WIWAM showed that GOLS2^{OE} plants have a reduced growth rate compared to WT plants under both control and mild drought conditions, resulting in a smaller rosette area and reduced yield. We therefore hypothesize that ectopic expression of GOLS2, and the thereby associated increased levels of RFOs, cause a (mild) yield penalty on plants. The yield (fresh weight) of GOLS2^{OE} plants is also reduced when grown in vitro (see Supplementary Figure 2*B*). If the assumption is true that RFOs stimulate ABA synthesis, increased ABA levels in GOLS2^{OE} plants might be responsible for the observed growth defects. In addition, the growth defect can be due to excessive and energy-demanding changes in carbohydrate metabolism. The yield penalty of GOLS2^{OE} plants was not reported before. Since the yield penalty is only mild, it could have been easily overlooked or it could be due to our growth conditions

CONCLUSIONS AND PERSPECTIVES

We have set-up a semi-automated system (WIWAM) that allows monitoring the growth of soil-grown plants under controlled watering conditions and this system has been tested on WT plants and two different transgenic lines, AVP1^{OE} and GOLS2^{OE}. Our results have shown that the system is operational and that it allows to reproduce the published enhanced growth phenotype of AVP1^{OE} plants. Additional experiments are being set-up to to evaluate the reproducibility of the system by running independent experiments on WT plants. Additional experiments are planned to validate the results for GOLS2 and more transgenic lines are in the pipeline to be tested. Transcriptome, metabolome and proteome analysis of drought-tolerant plants (i.e. plants with enhanced growth during drought) will be used to further elucidate the mechanisms that control growth during drought stress.

MATERIALS AND METHODS

Plant material, growth conditions and stress treatments

All experiments were carried out with seeds from wild type Col-0 and homozygous transgenic plants that were grown on the same tray in optimal growth conditions. For *in vitro* experiments, *Arabidopsis thaliana* seeds were sterilized by incubation with subsequently 70% ethanol (two minutes) and 5 % NaOCl (ten minutes). Plants were grown at 22°C and 65 µE m^{-2} s^{-1} radiation under continuous light conditions on MS medium containing 4.3 g/l Murashige-Skoog (MS; Duchefa, Haarlem, The Netherlands), 0.5 g/l MES, 10 g/l sucrose, 9 g/l plant tissue culture agar (LabM, Bury, UK). Before each experiment, stratification of seeds was done by incubation of the growth plates for three days at 4 °C in the dark.

Experiments with soil grown plants were conducted in a controlled growth chamber under a 16 h light / 8 h dark regime at 20 °C and 55 % relative humidity. For soil experiments, seeds were imbibed with water before sowing during three days at 4 °C and then sown with a pipetman® Neo (P1000; Gilson S. A. S., Villiers-le-Bel, France) directly onto the soil. From sowing to plant germination, the soil was humidified two times a day by spraying water. Between growth stage 1.02 and 1.04 (Boyes *et al*., 2001), plants of similar sizes were selected and thinned out to one plant per pot. For high-throughput screening of drought tolerance in soil, all plants were grown in separate pots on Jiffy-7 soil pellets (Jiffy Products, Norway). Plants were grown for 3.5 weeks under normal and manually controlled watering to ensure that all plants received a similar watering regime and that the water availability for each plant was comparable. This was achieved by bringing the total weight of each pot (plastic container, soil and plant) to 60-65 g with water on a regular basis (two-three times per week). After 3.5 weeks, all pots were brought to the target weight a last time and separated into two groups, one of which received further controlled watering (six plants per line), the other receiving no further watering (six plants per line). After 13 days of no watering, viability of the drought treated plants was scored, plants were rewatered and recovery was checked after 24 hours.

For comparing the growth of WT and transgenic plants under mild drought stress conditions, water-deficits were imposed by controlling and stabilizing the soil water status during development of soil-grown plants. Seed treatment, sowing and plant germination was performed as described above. Plants were germinated in cylindrical polypropylene pots (200 ml, Ø53, H88 mm, VWR International, REF 216-2648) filled with 90 g of soil (Saniflor professional potting compost containing 20 % organics; white peat, garden peat, fertilizer based on calcium and magnesium; pH 5.0-6.5; electric conductivity of 450 µS/n) at control soil water concentrations until growth stage 1.04 (Boyes *et al*., 2001). Then, wild type and transgenic plants were separated into three groups of

ten plants. One group was further grown under control conditions (1.75 g H_2O/ g dry soil), a second group was subjected to mild drought stress conditions (1.50 g H_2O/ g dry soil) and a last group was subjected to severe drought stress via a complete watering stop (until 0.00 g H_2O/ g dry soil).

Calculation of soil retention capacity

Soil water retention capacity was measured in a preliminary experiment. Pots were filled with soil, fully wetted and allowed to drain freely. Soil water content was determined by weighing the soil before and after drying (1 week and 65 °C). Soil water content at retention capacity (SRC) was ~5.5 g H_2O/ g dry soil.

Calculation of total target weight

The total target weight (TW_{Total}) for each sample was set as the sum of the empty pot (W_{Pot}), the amount of dry soil ($W_{dry\ soil}$) and the total amount of water (TW_{H2O}), which was calculated as the product of $W_{dry\ soil}$ and target soil water concentration (SWC). During the drought experiment, plants were watered every day so that the TW was reached and the soil water concentration stabilized.

$$TW_{Total}\ (g) = W_{Pot}\ (g) + W_{Dry\ Soil}\ (g) + TW_{H2O}\ (g)$$

$$TW_{H2O}\ (g) = SWC\ (gH_2O\ /\ g\ Dry\ Soil) * W_{Dry\ Soil}\ (g)$$

Rosette area quantification

During all stress experiments, pots containing one plant were recorded via digital imaging. These digital images were used for rosette area quantification with an in house developed script that allows automated analysis of high amounts of pictures (Roeland Merks). Images were first background-corrected with an in house developed image analysis software (based on the SDC Morphology Toolbox for MATLAB; http://www.mmorph.com). The program performs a RGB split and the blue color was retained to obtain a better contrast between plants and background. An arbitrary threshold was set to separate the plants from the background, the obtained images were then corrected for residual noise (based on the amount of joined pixels). The output is a comma separated file that gives the total amount of pixels per picture. These values were used to calculate the growth rate of the plants.

ACKNOWLEDGMENTS

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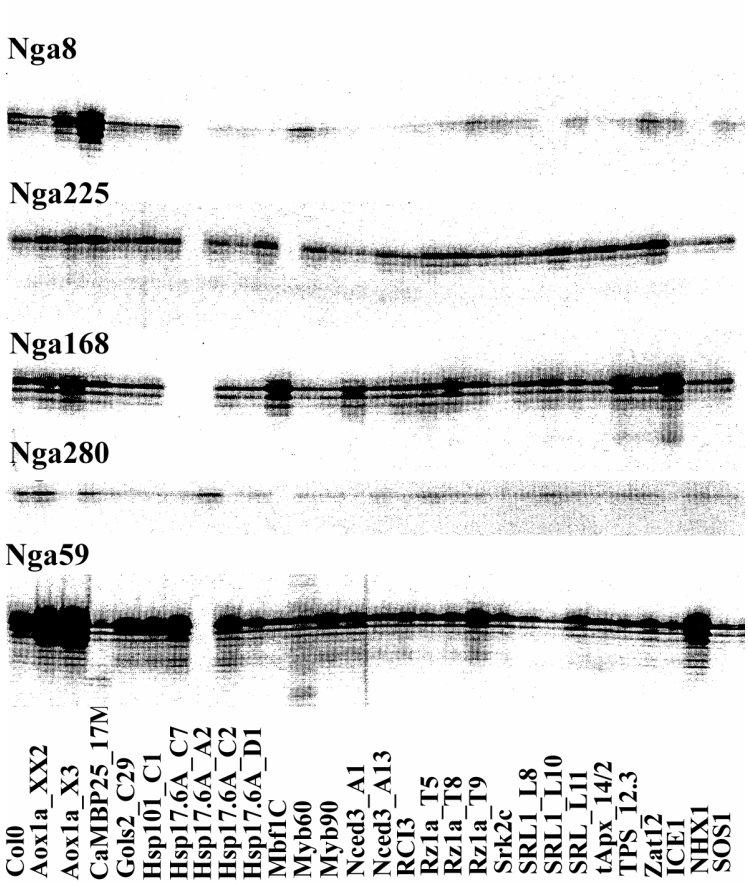
SUPPLEMENTARY DATA

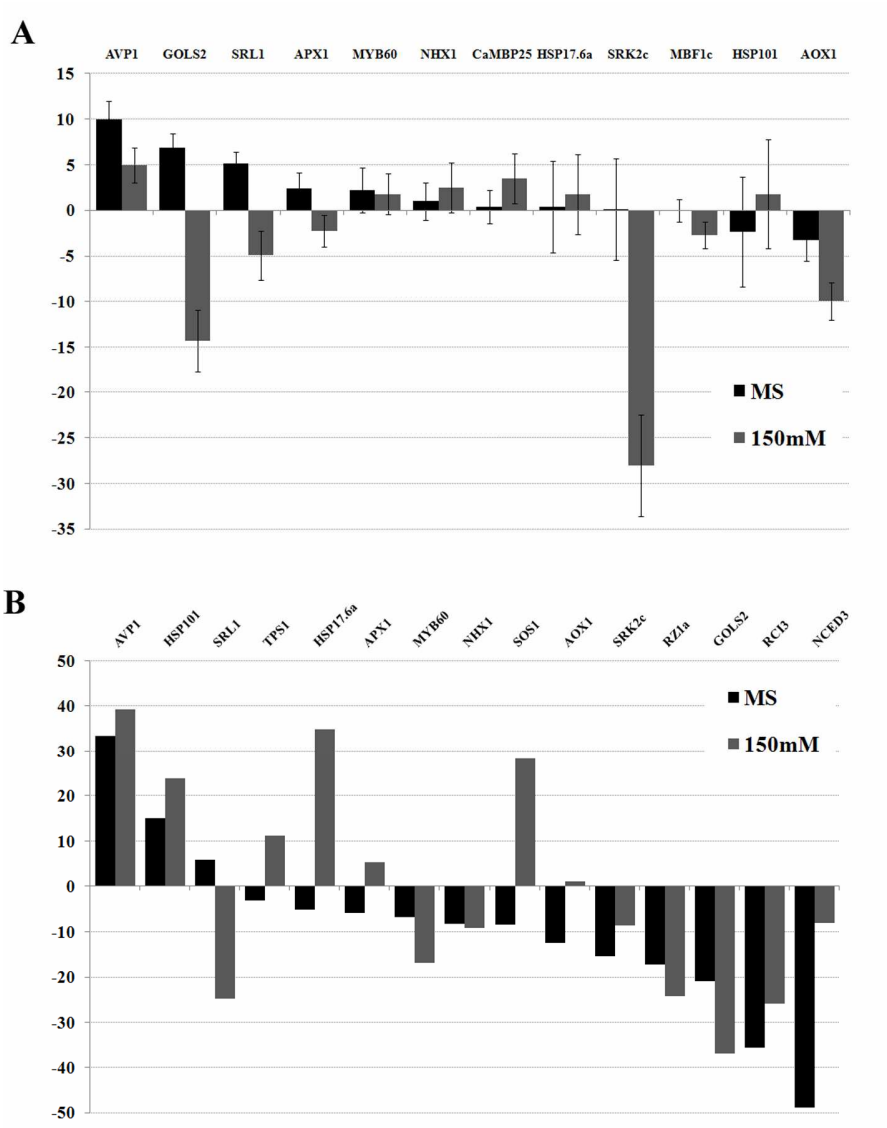
Detailed information on the received transgenic lines

Gene name	AGI code	line	Line_Nr	Selected lines	Ecotype confirmation	Homozygous
AOX1a	AT3G22370	XX-L	1	1.1-1.5	1.2	OK
		X-3	2	2.1-2.5	2.2	OK
AVP1	AT1G15690					received by Nathalie Gonzalez
CAMBP25	AT2G41010	17M	3	3.1-3.7	3.2	OK
GolS2	AT1G56600	C29	4	4.1- 4.5	4.2	OK
HSP101	AT1G74310	C1	5	5.1-5.6	5.2	OK
		C2	6	6.1-6.5	6.1	OK
HSP17.6A	AT5G12030	53.C7	7	7.1-7.5	7.2	OK
		58. A2	8	8.1	8.1	OK
		58.C2	9	9.1	9.1	OK
		58.D1	10	10.1,10.2	10.1	OK
MBF1c	AT3G24500	mbf1c	11	11.1-11.5	11.2	OK
MYB60	AT1G08810	myb60	12	12.1-12.5	12.2	OK
MYB90	AT1G66390	myb90	13	13.1	13.1	OK
NCED3	AT3G14440	A1	14	14.1-14.5	14.2	OK
		A13	15	15.1,15.2	15.2	OK
RCI3 (Rare Cold Inducible 3)	AT1G05260	rcl3OE	16	16.1-16.4	16.2	OK
RZ-1a	AT3G26420	T5	17	17.1-17.3	17.1	OK
		T8	18	18.1-18.5	18.1	OK
		T9	19	19.1	19.1	OK
SRK2C	AT1G78290	SRK2C-GFP	20	20.1-20.5	20.1	OK
SRL1	AT5G37370	L4	21			not OK
		L7	22			not OK
		L8	23	23.1	23.1	not OK
		L10	24	24.1	24.1	not OK
		L11	25	25.1	25.1	not OK
tAPX	AT1G77490	14/2	26	26.1-26.6	26.2	OK
TPS1	AT1G78580	12.3	27	27.1-27.7	27.2	OK
Zat12	AT5G59820		28	28.1-28.5	28.2	OK
ICE1	AT3G26744		29	29.1-29.5	29.1	OK
NHX1	AT5G27150		30	30.1-30.5	30.1	OK
SOS1	AT2G01980		31	31.1-31.5	31.1	OK



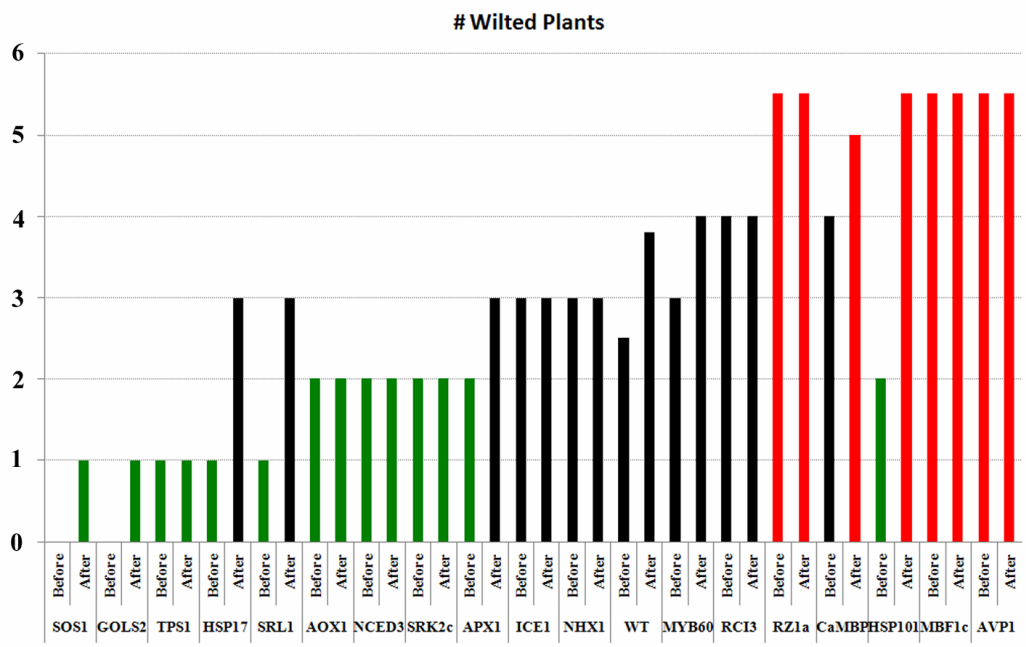
Supplementary Figure 1

Microsatellite analysis on transgenic lines. Microsatellite analysis was performed on genomic DNA (gDNA) that was extracted from three week old plants using 1% CTAB (Hexadecyl Trimethyl Ammonium Bromide, Sigma®) buffer (0.1M Tris-HCl pH7.5, 0.7M NaCl, 0.01M EDTA). Fluorescent labeled microsatellite primers (IRD_{700}) for the following markers were used: nga8, nga59, nga168, nga225 and nga280 (sequences can be obtained from http://www.arabidopsis.org/). One microliter of the gDNA (2ng/µl) was added to 20 µL of polymerase chain reaction (PCR) mixture (0.25 mM dNTPs, PCR reaction buffer, 1.5 mM $MgCl_2$, 0.375 µM reverse primer, 0.2-2.5 pmol IRD_{700} primer and 1.2 units of Taq). These reactions were then run with the following cycle program: 94 °C for 1 min; 40 cycles of 94 °C for 15 sec, 56 °C for 20 sec, and 72 °C for 30 sec; 72 °C for 2 min; and 4 °C final. The polymorphisms were separated using gel-electrophoresis and visualized with LICOR. Each marker resulted in a specific pattern for Columbia (Col) and lines with different patterns were regarded as genetically different. All received lines were in a Col background.



Supplementary Figure 2.

Data from the mannitol screen. One week-old plants were transferred from vertical MS plates to MS plates containing 0 and 150 mM mannitol. Root length was recorded during one week. Then plants from the same genotype were pooled per treatment and fresh weight was measured. A. Increase or decrease in root length compared to WT *Arabidopsis* plants. B. Increase or Decrease in FW compared to WT *Arabidopsis* plants.



Supplementary Figure 3

Data from the soil drying screen for enhanced drought tolerance. Per Genotype, six plants (3.5 week-old) were withhold from watering for two weeks, rewatered and allowed to recover for one day. The numbers of wilted plants were scored. Green bars indicate resistant lines, red bares represent sensitive lines.

CHAPTER 6

Molecular phenotyping of drought tolerant *Arabidopsis* plants overexpressing a galactinol synthase, GOLS2, reveals a role for *myo*-inositol

ABSTRACT

The response of plants to drought stress is controlled via drastic changes in gene expression, hence microarrays are a useful tool to decipher the molecular mechanisms that control tolerance. Here, we report the molecular phenotype of stress-tolerant *Arabidopsis* plants with increased levels of GOLS2. GOLS2 encodes a galactinol synthase that is involved in osmoprotection of cellular structures during stress. Ectopic expression of GOLS2 affected the stress response of plants and resulted in decreased expression of drought-induced genes. Furthermore, the microarray analysis of GOLS2^{OE} plants also indicated that the expression of genes of which the products are involved in carbohydrate metabolism was affected. MIOX2, encoding a key enzyme in the *myo*-inositol (MI) oxygenation pathway, was most strongly induced by GOLS2 overexpression. The MI oxygenation pathway is an alternative route for the production of cell wall precursors. Our microarray data indicates that ectopic expression of GOLS2 *in Arabidopsis* does not only alter the carbon partitioning between sucrose and raffinose, but also redirects MI metabolism towards cell wall biosynthesis.

INTRODUCTION

Plants have adapted to respond to drought stress at the molecular level by inducing the expression of defense genes, which enables them to survive (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 2007). Microarray analysis has contributed significantly to the current understanding of the molecular response of plants to drought stress (Catala *et al*., 2007; Oono *et al*., 2003; Oztur *et al*., 2002; Rabbani *et al*., 2003; Seki *et al*., 2001a; Seki *et al*., 2001b). The products of stress-induced genes not only function in stress tolerance (functional or single action genes), but also in signal amplification (regulatory genes) of the stress response (Shinozaki and Yamaguchi-Shinozaki, 2007). Nowadays, much attention is drawn on the regulatory mechanisms controlling the stress response of plants by functional analysis of stress-inducible transcription factors (TFs). Stress-inducible TFs can regulate common gene sets and thereby can confer tolerance to multiple stresses, including drought, salt, cold and freezing stress, indicating that an extensive cross-talk must exist. The expression of stress-inducible TFs can be controlled via abscisic acid (ABA)-dependent or ABA-independent pathways (Yamaguchi-Shinozaki and Shinozaki, 2006; Shinozaki and Yamaguchi-Shinozaki, 2007). The best described stress-responsive TFs are the C-repeat-binding factor (CBF)/dehydration-responsive element-binding (DREB) proteins that belong to the AP2/ethylene-responsive element binding protein family (Maruyama *et al*., 2004; Gilmour *et al*., 2004). CBF/DREB1 expression is upstream controlled by ICE1 (inducer of CBF expression 1), a MYC-like bHLH TF (Chinnusamy *et al*., 2003). Downstream regulated genes include TFs, such as ZAT10/STZ, that are involved in further regulation of the stress response. CBF/DREB proteins can induce tolerance to multiple stresses, including drought, salt, cold and freezing tolerance (Haake *et al*., 2002; Jaglo-Ottosen *et al.*, 1998; Kasuga *et al*., 1999; Novillo *et al*., 2004). Other important stress-responsive TFs include the ABA-responsive element (ABRE)-binding factors (ABFs), MYC2, MYB2, RD26/NAC (Abe *et al*., 2003; Tran *et al*., 2004). Ectopic expression of ABFs, which belong to the bZIP protein family, resulted in improved tolerance to drought stress by regulating the expression of ABA-responsive genes (Fujita *et al*., 2005; Kang *et al*., 2002; Kim *et al*., 2004). The importance of transcriptional networks during drought responses was underscored by a recent report stating that approximately 40 *Arabidopsis* TFs, at least some with novel functions, can enhance tolerance to drought stress (Nelson *et al*., 2007).

TFs induce the expression of single action genes to confer tolerance to stress (Umezawa *et al*., 2006). Such single-action genes can be involved in different processes, including ion transport and biosynthesis of protective metabolites (see Chapter 1). The expression of genes involved in the biosynthesis of protective metabolites, such as raffinose family oligosaccharydes (RFOs), during cold and drought stress is controlled by by CBF/DREB1 TFs (Fowler *et al*., 2002; Maruyama *et al*., 2004;

Seki *et al*., 2001a; Vogel *et al*., 2005). Accumulation of RFOs during cold and drought stress protects cells from the effects of dehydration and accordingly, transgenic plants that produce more RFOs, for example by overexpression of galactinol synthase (GOLS2, AT1G56600), showed enhance stress tolerance (Taji *et al*., 2002; Chapter 5). GOLS2 catalyzes the first step in RFO synthesis (the production of galactinol) and plays a key regulatory role in the carbon partitioning in plants by using two important sugars, *myo*-inositol (MI) and sucrose, as substrates. Induction of genes encoding ion transporters, such as salt overlay sensitive 1 (SOS1, AT2G01980), is important for re-establishing ionic homeostasis and this is especially relevant during high salinity. Ectopic expression of SOS1 in plants increased tolerance to salt stress (Shi *et al*., 2003).

The results presented here are part of an ongoing project of which the aim is to elucidate the mechanisms that control plant growth during drought stress by analyzing the molecular phenotypes of drought-tolerant transgenic *Arabidopsis* plants. In chapter 5, it was described how stress-tolerant transgenic *Arabidopsis* lines were selected and evaluated for drought tolerance. Here, the transcriptome of $SOS1^{OE}$ and $GOLS2^{OE}$ plants was analyzed using microarrays.

RESULTS AND DISCUSSION

Microarray analysis on $SOS1^{OE}$ and $GOLS2^{OE}$ transgenic plants

We used gene expression data as molecular phenotype to obtain further insights into the downstream effects provoked by perturbation of SOS1 and GOLS2 expression in *Arabidopsis* (Columbia-0, Col-0). Therefore, a genome-wide microarray analysis was performed on wild type (WT) Col-0, and $SOS1^{OE}$ and $GOLS2^{OE}$ transgenic plants. The transcriptome of $SOS1^{OE}$ and $GOLS2^{OE}$ plants was compared with that of WT plants under non-stressed conditions at developmental stage 1.04 (Boyes *et al*., 2001). At this stage, seedlings contain both dividing and developing tissues thereby providing a mix of genes that are expressed in different developmental programs. No obvious phenotypical differences were detected between WT and transgenic plants at this stage. Per genotype, three independent samples of ten pooled seedlings grown *in vitro* were harvested. RNA was isolated and hydridized to full-genome Affymetrix® ATH1 Genechip® microarrays. Probe sets with a *p*-value smaller than 0.05 after multiple testing correction were considered as differentially expressed. For $SOS1^{OE}$ and $GOLS2^{OE}$, approximately 1600 and 700 probe sets, respectively, were differentially expressed. For further analysis, only those probe sets with a fold change (FC) expression greater than two were retained, unless mentioned otherwise.

An overview of the numbers of probesets affected in $SOS1^{OE}$ and $GOLS2^{OE}$ lines is shown in table 1. Final lists for the induced and repressed probesets in $SOS1^{OE}$ and $GOLS2^{OE}$ can be found in Supplementary Table S5 and Supplementary Table S6, respectively.

Table 1. Number of probesets with a FC higher than 2

Gene	Profile	Probesets
GOLS2	Up	62
	Down	34
SOS1	Up	141
	Down	107

BiNGO analysis (Maere *et al*., 2005) on SOS1 downregulated genes indicated an overrepresentation of genes involved in lipid metabolism, which were found within the genes with the highest fold reductions (data not shown). However, it was later noticed that fully developed $SOS1^{OE}$ plants did not produce trichomes. Our hypothesis is that the $SOS1^{OE}$ construct might have been transformed in a trichome-defective mutant background, such as GLABROUS (Larkin *et al*., 1994), or that the transgene has disrupted some component of the trichome pathway. Since WT Col-0 was used as control for the microarray experiment, we consider these results as possible artifacts. This hypothesis is strengthened by the fact that the SOS1 transcript is not stable under non-stressed conditions and only accumulates to high levels upon NaCl treatment (Shi *et al*., 2003; Chung *et al*., 2008). We further focus on the microarray data for GOLS2. Quantitative real-time PCR data confirming the expression of GOLS2-dependent genes is shown in Figure 1.

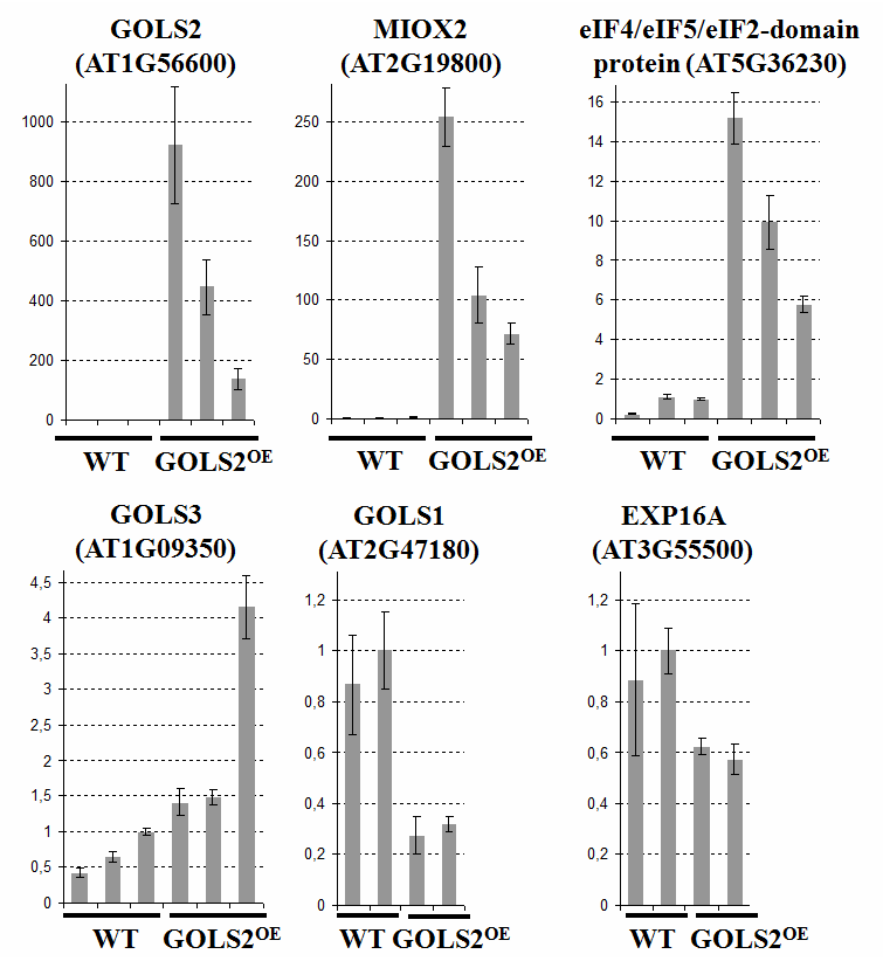


Figure 1

Relative expression data for GOLS2-dependent genes. Quantitative RT-PCR was performed on three independent samples for WT and GOLS2^{OE} plants that were harvested from a biological repeat experiment. Errors bars represent standard error of three technical repeats.

Ectopic expression of GOLS2 leads to repressed of drought-induced genes

Genevestigator® was used to study the stress-related expression of the 96 genes that were up- or downregulated in GOLS2^{OE} plants (Figure 2; Zimmermann *et al*., 2005). The expression of approximately 80 % of these 96 genes was affected by stress. Two subclusters, A and C, contained genes that are upregulated by drought, salt, osmotic, heat, cold or ABA treatments, while subcluster B contained genes that were downregulated by these stresses. Oxidative and genotoxic stress did not change the expression of the GOLS2-deregulated gene set. The altered stress response of GOLS2^{OE} lines was independently confirmed by BiNGO analysis for overrepresentation of certain GO labels, which included “response to abiotic stress” (data not shown, Maere *et al*., 2005).

To specifically study the relation between the drought tolerant phenotype of GOLS2^{OE} transgenic plants and their molecular phenotypes, we investigated whether known drought-inducible genes are affected in these lines. We used a set of 1686 genes that were induced (FC > 2) by drought stress in WT *Arabidopsis* plants (Catala *et al*., 2007). The hypothesis was that constitutive activation of drought-responses might contribute to the stress tolerance of GOLS2^{OE} plants. Overall, 21 drought-inducible genes were identified within the GOLS2-dependent genes (FC > 2), but no relevant constitutive induction of drought-induced genes was found in GOLS2^{OE} plant. Surprisingly, 17 of the 34 GOLS2-downregulated genes were strongly induced by drought (Table 2). These genes encode proteins that are involved in redox control (e.g. ferritin, flavin-containing monooxygenase), cell wall modifications (expansin, lipid transfers proteins), defense responses (heat shock protein 70, GOLS1) and several biosynthetic pathways (methionine gamma-lyase, 3-phosphoglycerate dehydrogenase). It remains to be investigated why these drought responsive genes are downregulated by GOLS2 overexpression. In the drought experiment performed by Catala and coworkers (2007), rosettes were detached from the roots and allowed to dry for two hours (Catala *et al*., 2007). Such a drastic treatment might have resulted in the induction of genes that were responsible for secondary drought effects such as wilting or growth inhibition, rather than genes involved in drought tolerance, which might explain why such genes are downregulated in drought-tolerant GOLS2^{OE} plants.

To assess whether the mechanism of drought tolerance in GOLS2^{OE} transgenic plants is novel, comparisons were made with the gene expression profiles from ABA-treated wild type plants and from CBF4 TF-overexpressing plants, each of which have well known drought-tolerance genes induced, and also with the profiles from a recently identified TF, NF-YB, which works independently from ABA and CBF4 (Haake *et al*., 2002; Nelson *et al*., 2007). However, no significant overlap was observed between GOLS2 regulated genes and genes regulated in response to ABA treatment nor with those expressed in CBF4^{OE} plants (which show strong correlation with known drought/stress-response pathways) or in NF-YB^{OE} plants. Comparison with the profiles from other known stress tolerant transgenic lines, including CBF2^{OE}, MBF1c^{OE}, ZAT12^{OE}, MKK2^{OE}, did not point to a relevant overlap with the GOLS2^{OE} and SOS1^{OE} profiles. This suggest that the drought tolerance mechanisms downstream of GOLS2 do not involve TFs or other regulatory genes with known functions during tolerance to abiotic stress.

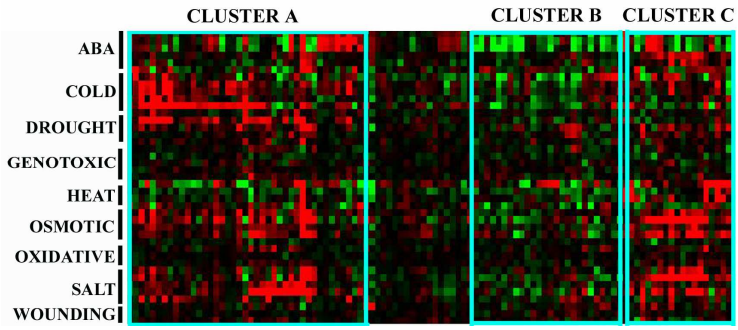


Figure 2

Stress-related expression of GOLS2-dependent genes. For all genes, expression data on ABA and diverse abiotic stress treatments (cold, drought, genotoxic, heat, osmotic, oxidative, salt and wounding) was clustered and visualized using the stimulus profiles in Genevestigator® (Zimmermann *et al*., 2005). Expression of different genes during the same treatment are visualized on the horizontal axes and the expression of one STGs after different stress treatments is shown vertically. Three subgroups were distinguished (Cluster A-C). Red colors indicate induction, green colors represents repressed genes. Multiple experiments for the same stress treatment are indicated on the left by vertical black bars. Red colors indicate induction, green colors represent repressed genes.

Table 2. Drought-induction of GOLS2-repressed genes

AGI	Descripiton	FC GOLS2^{OE}	FC Drought
AT5G59310	LTP4 (LIPID TRANSFER PROTEIN 4); lipid binding	-2.83	75.51
AT2G37770	aldo/keto reductase family protein, oxidoreductase activity	-2.11	57.50
AT2G37870	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	-3.41	33.71
AT3G12580	HSP70 (heat shock protein 70); ATP binding	-2.19	31.26
AT2G33380	RD20 (RESPONSIVE TO DESSICATION 20); calcium ion binding	-3.41	27.98
AT1G62540	flavin-containing monooxygenase family protein / FMO family protein	-2.53	22.20
AT3G55500	ATEXPA16 (*ARABIDOPSIS THALIANA* EXPANSIN A16)	-2.85	22.02
AT2G47180	ATGOLS1 (*ARABIDOPSIS THALIANA* GALACTINOL SYNTHASE 1); transferase, transferring hexosyl groups	-2.51	21.50
AT1G64660	ATMGL; catalytic/ methionine gamma-lyase	-2.55	7.91
AT3G28270	similar to protein of unknown function	-2.07	7.60
AT1G17745	PGDH (3-PHOSPHOGLYCERATE DEHYDROGENASE); phosphoglycerate dehydrogenase	-2.06	7.16
AT2G32990	glycosyl hydrolase family 9 protein	-2.71	5.39
AT3G51240	F3H (TRANSPARENT TESTA 6); naringenin 3-dioxygenase	-2.42	3.60
AT5G01600	ATFER1 (ferretin 1); ferric iron binding	-3.57	2.93
AT2G22240	inositol-3-phosphate synthase isozyme 2 / IPS 2	-2.11	2.75
AT1G52030/ AT1G52040	[AT1G52030, MBP2 (MYROSINASE-BINDING PROTEIN 2)];[AT1G52040, MBP1 (MYROSINASE-BINDING PROTEIN 1)]	-2.03	2.74
AT2G21560	similar to unknown protein	-2.15	2.32

GOLS2^{OE} affects the expression of genes involved in *myo*-inositol metabolism

PageMan software, which allows to annotate microarray data in the context of functional ontologies and to statistically validate over-representation of functional classes, was used to get insight into the metabolic processes affected by GOLS2^{OE} (Usadel *et al*., 2006). PageMan analysis of all genes that were significantly (*p*-value < 0.05) deregulated in GOLS2^{OE} plants pointed towards altered carbohydrate (CHO) metabolism. MapMan software, which is complementary to PageMan, was further used for the visualization in diagrams of gene expression changes of individual genes, with genes grouped by function or class (Thimm *et al*., 2004). In the resulting MapMan diagram for *Arabidopsis* metabolism, CHO (minor CHO, starch and sucrose) metabolism was indeed strongly affected in GOLS2^{OE} plants (Figure 3). Table 3 lists genes with a FC higher than two and for which the encoded proteins have known functions in CHO (e.g. raffinose, galactinol, MI) or starch metabolism. However, Table 3 does not reflect the total number of affected carbohydrate metabolic genes as many more genes are differentially expressed with a FC lower than 2.0 (data not shown).

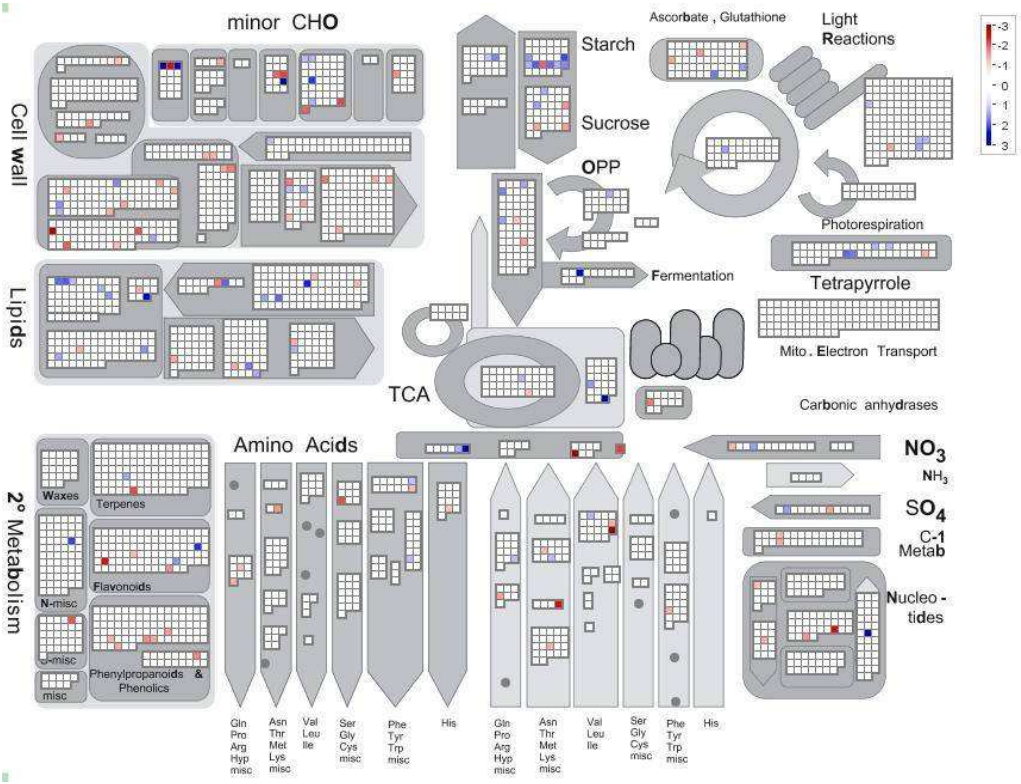


Figure 3

MAPMAN visualization of metabolic pathways in GOLS2^{OE} plants. Genes were assigned to their associated metabolic pathway. Genes that were differentially expressed (*p* value < 0.05) are visualized. Blue, red, and white boxes represent repressed, induced, and genes that were not differentially expressed, respectively.

Table 3. Differential expressed (FC > 2) carbohydrate (CHO) metabolic genes in GOLS2^{OE} plants

AGI	Description	Pathway	FC GOLS2^{OE}
AT1G56600	GOLS2 (Galactinol Synthase 2)	Minor CHO	109.17
AT2G19800	MIOX2 (Myo-inositol oxygenase 2)	Minor CHO	54.13
AT5G62360	Invertase/ pectin esterase inhibitor	Minor CHO	7.55
AT5G26340	MSS1 (SUGAR TRANSPORT PROTEIN 13)	Minor CHO	3.63
AT1G09350	GOLS3 (Galactinol Synthase 3)	Minor CHO	2.68
AT3G47800	Aldose 1-epimerase family protein	Minor CHO	2.19
AT3G46970	Alpha-glucan phosphorylase	Starch	2.07
AT2G22240	Inositol-3-phosphate synthase isozyme 2 (IPS 2)	Minor CHO	-2.11
AT2G37770	Aldo/keto reductase family protein	Minor CHO	-2.11
AT2G47180	GOLS1 (Galactinol Synthase 1)	Minor CHO	-2.51
AT2G32990	Glycosyl hydrolase family 9 protein	Minor CHO	-2.71

Expression of MI oxygenase 2 (MIOX2), which is involved in the MI-oxygenation pathway, was more than 50-fold induced in GOLS2^{OE} plants. No genes downstream of MIOX2 in the MI oxygenation pathways were differentially expressed in GOLS2^{OE} plants. In contrast, overexpression of GOLS2 affect the expression of two other genes encoding galactinol synthase enzymes, GOLS1 and GOLS3 (Figure 4).

MI and MI-derived products play a central role in plant growth and development, with functions in the biogenesis of membranes and cell walls, production of RFOs, formation of auxin conjugates, nutrient storage, signal transduction and response to stress (Loewus and Murthy, 2000). The fact that MIOX2 was the highest induced in GOLS2^{OE} plants is of particular interest since GOLS2 and MIOX2 both use MI as substrate (Figure 4). GOLS2 catalyzes the first committed step in the biosynthesis of RFOs, and plays a key regulatory role in the carbon partitioning between sucrose and RFOs. MIOX2 is the key enzyme in the *myo*-inositol oxygenation pathway and catalyzes the oxygenative cleavage of MI to a nucleotice sugar, glucuronic acid (GlcA), which is a precursor for UDP-GlcA, the most important precursor for cell wall matrix polysaccharides. The production of UDP-GlcA is irreversible and UDP-GlcA can thus not be reconverted into MI or other carbohydrate storage compounds (Kanter *et al*., 2005). The combination of increased levels of GOLS2 and MIOX2 in GOLS2^{OE} plants pushes MI towards the production of RFOs and cell wall components, thereby aiding stress defense responses. Two inositol-phosphate synthase (IPS) genes that are responsible for the first committed step in MI biosynthesis, which is the production of MI-1-phosphate (MI-1-P) from glucose-6-phosphate (Glc-6-P), were downregulated and it can be assumed that this, together with increased MIOX2 levels would result in a depletion of MI (Figure 4).

We previously showed that GOLS2^{OE} plants showed a decreased growth compared to WT plants (see Chapter 5). The growth defect in GOLS2^{OE} plants might be an indirect effect of exuberant energy consumption due to increased metabolic rates. It is possible that overexpression

of GOLS2 and subsequent increase in raffinose production causes sucrose depletion. The upregulation of the putative invertase (AT5G62360), which mediate cleavage of sucrose into hexose monomers, and the hexose transporter (AT5G26340) could contribute to sucrose starvation in GOLS2^{OE} plants (Table 3; Roitsch and González, 2004). Because of its importance as storage molecule, it is possible that, next to sucrose starvation, also depletion of MI (and MI-derived components such as MI-1-P) is responsible for the negative growth affect of GOLS2^{OE} plants. If this hypothesis is true, it would not explain why MIOX2 is induced in carbon-starved plants (Osuna *et al*., 2007), for the reason that increased catabolism of MI would then aggravate the effect of carbon starvation. Alternatively, the yield penalty of GOLS2^{OE} plants can be a result of altered auxin physiology. Free MI is a substrate for auxin-ester conjugates (MI-IAA), which serve as auxin storage forms or as auxin transport intermediates. However, the exact function of MI-IAA is unknown.

Induction of MIOX2 expression levels by GOLS2 overexpression might be caused by an upstream signal to decrease growth and nutrient usage during nutrient-limiting conditions, for example by stimulating MI catabolism towards cell wall biosynthesis, leading to increased cell wall thickness and inhibition of cell elongation (two expansins are downregulated in GOLS2^{OE} plants). The growth reduction of GOLS2^{OE} plants would then not be an indirect effect of high metabolic rates, but would be caused by a direct signal to allow the plant to survive under drought stress conditions.

PERSPECTIVES

Future work will focus on the relevance of MIOX2 induction in GOLS2^{OE} plants and the role of the MIOX2 gene during stress tolerance. Knock-out mutants for MIOX2 (miox2^{KO}) lines have been requested and these will be tested by using various drought stress assays. Furthermore, the miox2 mutation will be incorporated in GOLS2^{OE} plants and the effect on growth under normal and drought stress conditions will be evaluated. This will allow to investigate if the induction of MIOX2 in GOLS2^{OE} plants is necessary for the observed phenotypes. Furthermore, it would be very interesting to investigate the metabolic changes occurring in GOLS2^{OE} and GOLS2^{OE}miox2^{KO} plants.

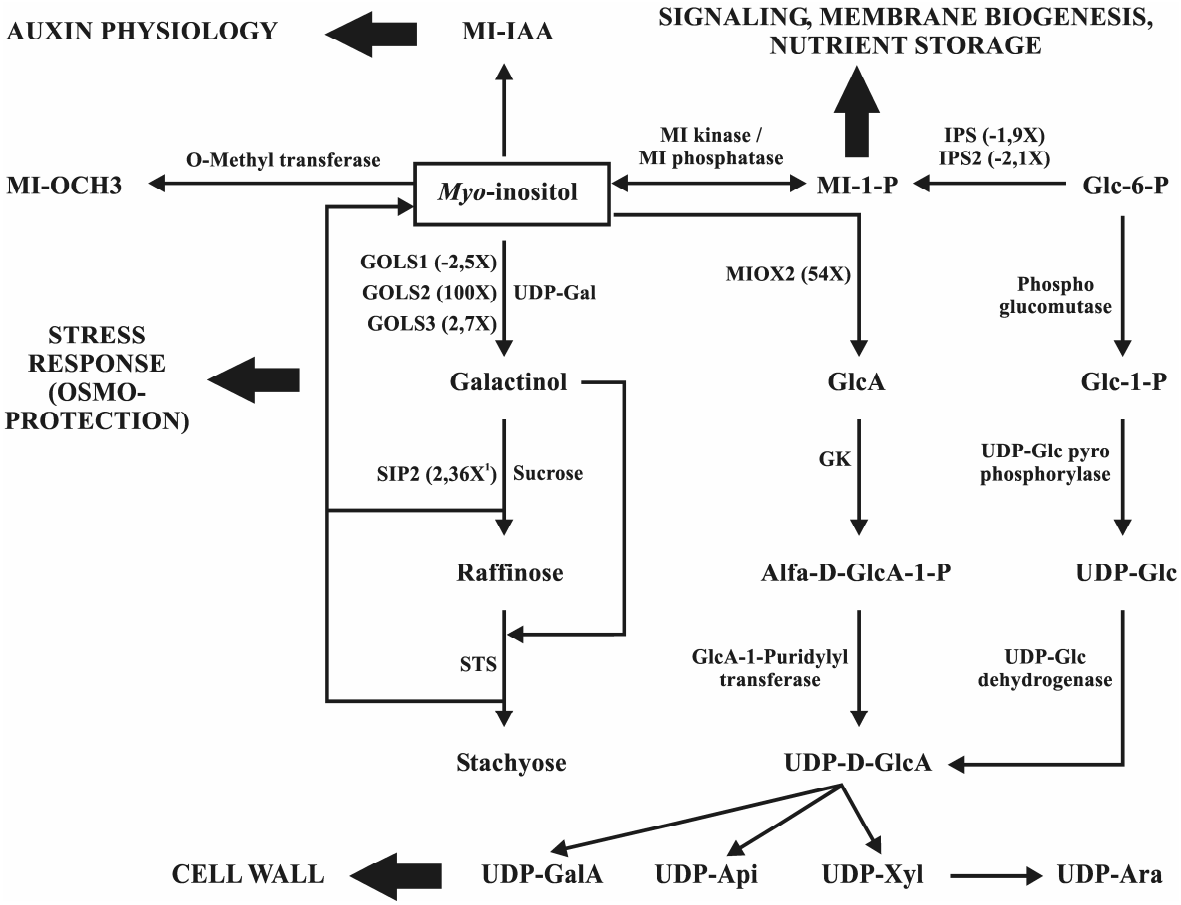


Figure 4

Myo-inositol metabolic pathway. Expression of genes in red are induced and that of genes in green are repressed in GOLS2^{OE} plants. Genes in black were not differentially expressed. Fold changes are shown between brackets. [1]gene is induced in GOLS2^{OE} plants, but not significant (*p* value > 0.05) due to strong variations in the technical repeats. STS, stachyose synthase; IAA, indol-acetic acid; Glc, glucose; GlcA, glucoronic Acid; GK, glucoronokinase; UDP, uridine diphosphate, Glc-1-P, glucose-1-phosphate; GalA, galacturonic acid; Api, apiose; Xyl, xylose, Ara, arabinose. Thin arrows indicate enzymatic reactions and thick arrow represent general functions for the end products of certain enzymatic reactions.

MATERIALS AND METHODS

Microarray Analysis

All experiments were carried out with seeds from wild type Col-0 and homozygous transgenic plants that were grown on the same tray in optimal growth conditions. For *in vitro* experiments, *Arabidopsis thaliana* seeds were sterilized by incubation with subsequently 70% ethanol (two minutes) and 5 % NaOCl (ten minutes). Plants were grown at 22°C and 65 µE m^{-2} s^{-1} radiation in a 16-h-light/8-hdark photoperiod on MS medium containing 4.3 g/l Murashige-Skoog (MS; Duchefa, Haarlem, The Netherlands), 0.5 g/l MES, 10 g/l sucrose, 9 g/l plant tissue culture agar (LabM, Bury,

UK). Seedlings were harvested at growth stage 1.04 (Boyes *et al*., 2001) in three independent biological repeats per genotype so that each sample represented a pool of ten plants obtained from three different growth plates. Total RNA was isolated using Qiagen commercial products (RNeasy® Plant Mini Kit, Cat. N° 74904) according to the manufacturer's instructions. The concentration and quality of total RNA was determined with a Nanodrop ND-1000 spectrophotometer (Agilent Technologies, Palo Alto, CA), so that fulfilled the required standards of the VIB microarray facility (MAF; Leuven, Belgium), were further RNA handling and Affymetrix® chip analyses were performed under the manufacturer's conditions for reverse transcription, labeling, hybridization, and scanning (https://www.affymetrix.com). Each triplicate of *Arabidopsis* Col-0 and homozygous transgenic plants was hybridized to one Affymetrix® chip (Genechip® *Arabidopsis* ATH1 Genome Array; Affymetrix, Santa Clara, CA). For each hybridization, 5 µg of total RNA was used. Raw data was processed via the Affylmgui software package, which is available through R-software. Significance estimation of the observed differences for each probeset was done using BH statistics (Benjamini and Hochberg, 1995).

Quantitative RT-PCR

For the confirmation of the microarray data, transcript levels were quantified using real-time PCR. In an independent experiment, plants were grown as described above and total RNA was prepared using TRIzol Reagent (Invitrogen, Carlsbad CA) and cleaned using Qiagen commercial products (RNeasy® Mini Kit, Cat. N° 74106) was used for RNA clean-up. The concentration and quality of total RNA was determined with a Nanodrop ND-1000 spectrophotometer (Agilent Technologies, Palo Alto, CA), and 1 µg of total RNA was reverse-transcribed with Superscript II RNAseH$^-$ Reverse Transcriptase, followed by an RNAse treatment according to the manufacturers' instructions (Invitrogen, Carlsbad CA). First-strand cDNA was used as a template in a subsequent PCR, which was performed on the iCycler IQ (Bio-Rad, Hercules, CA). The transcripts were amplified using gene-specific primers (AT1G56600-FWD: TCGGTTATTGCCAACAGTG; AT1G56600-REV: GAGGTTATGATAAGTGGAGAGG ; AT2G19800-FWD: GACAGAGATGATCTCAAGTGG; AT2G19800-REV: CGCCGGAAAATACTTGTTGATG; AT3G55500-FWD: GTTTTCCTCAAGATCGCTGAG; AT3G55500-REV: CGTTCGTAATCAGCACCAAG; AT2G47180-FWD: GACTCCTTTCGCTGAACAGG; AT2G47180-REV: CAGTAGTGAACCACCTTGAC; AT5G36230-FWD: TTCCCAGAAGTAGTGAGGTCG; AT5G36230-REV: AGGTTTGCCTGCCCTTTGAG; AT1G09350-FWD: CCACACCTTTTGCTGAACAG; AT1G09350-REV: GTGAACCTCCAAGGCTTAGC).

BiNGO analysis

The BiNGO plugin for Cytoscape (Agilent technologies) was used to look for overrepresentation of GO classes in our selected genes (Maere *et al*., 2005). Hypergeometrix testing was done with Benjamini and Hochberg False Discovery Rate (FDR) correction (Benjamini and Hochberg, 1995). Overrepresented categories were visualized based on *p* values larger than 0.05 and using all the genes present on the ATH1 array as reference set. Obscure evidence codes, including IEA (inferred from electronic annotation), NR (not recorded), NAS (non-traceable author statement), were discarded. For more information, see http://www.psb.ugent.be/cbd/papers/BiNGO/.

Genevestigator analysis

Within the different tools of Genevestigator, Stimulus Viewer was used to reveal the response profiles of genes to different stimuli (Zimmermann *et al*., 2005). Out of the different conditions annotated, the abiotic stresses (Kudla's Laboratory, Germany) and hormone treatments (Yoshida's Laboratory, Japan) were chosen for comparison with our selected differential expressed genes. For more information, see https://www.genevestigator.ethz.ch.

Pageman and MapMan analysis

PageMan analysis with our selected genes and using all the genes present on the ATH1 array as reference set was performed on http://mapman.mpimp-golm.mpg.de/general/ora/ora.html. MapMan software, pathways and mappings was downloaded from https://gabi.rzpd.de/projects/MapMan/.

Supplementary Tables

Supplementary Tables for this chapter are included as addendum at the back of thesis and are also made available in an electronic version on the attached compact disc.

Supplementary Table S5. SOS1-dependent genes

Supplementary Table S6. GOLS2-dependent genes

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PART IV

GENERAL DISCUSSION AND SUMMARY OF THE THESIS

CHAPTER 7

GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES

The response of plants to environmental stimuli

An important part of my Ph.D. dealt with the response of plants to environmental stresses. In the last decade, it has become evident that the stress response of plants consists of an extensive signaling network that is largely controlled by stress-inducible defense proteins. From the current literature, a number of interesting aspects of plant stress responses are apparent: Reactive oxygen species (ROS) play a central role in the stress response of plants; transcription factors and other signaling proteins are excellent candidates to improve the stress and defense response of plants (so called regulon approach); transcriptome analysis can provide insight into the molecular mechanisms that controls the stress and defense response of plants; and modifying the stress response of plants can result in a yield penalty.

Hydrogen peroxide (H_2O_2) as powerful signaling molecule during plant stress

An important aspect within the (early) response of plants to (almost) all stresses is the accumulation of ROS. Plants that can respond to and cope with increased ROS levels are often protected against the detrimental effects of the stress. ROS, including H_2O_2, are now also considered to be central regulators of the response of plants to environmental stresses. This has been confirmed by a high number of transcriptome studies showing that H_2O_2 alters the expression of many defense genes. The general aim of my thesis was related to the main research activities of my research group which tries to unravel H_2O_2-signaling in plants. A first contribution of this thesis to the current understanding of H_2O_2 signaling in plants concerned the evolutionarily conservation of the transcriptional response to increased H_2O_2. The results from this work can be explored using an on-line tool and we believe it can be of future value for other researchers that want to check their genes of interest. Moreover, the existing platform could be used to include additional transcriptome data if desired. Because of the central role of H_2O_2 as signal molecule during stress, we hypothesized that H_2O_2-induced genes could serve a candidates for improvement of stress tolerance in plants. A second contribution of this thesis to the current knowledge on H_2O_2 signaling in plants was delivered by the evaluation of stress tolerance of plants with altered expression levels of H_2O_2-induced genes. At this point, we gave priority to H_2O_2-induced genes, with a focus on genes

with (putative) regulatory functions, that were identified by previous transcriptome analysis in the research group. Two different functional screens (one for biotic stress and one for oxidative) were performed and both resulted in a number of H_2O_2-induced genes with putative functions during the stress response of plants. In our opinion, the most important finding from both functional screens was that overexpression of the WRKY15 transcription factor increased tolerance to H_2O_2-induced oxidative stress. These results were followed-up in more detail by phenotypic analysis of WRKY15 overexpressor lines during several abiotic stresses. As drought stress is (or will become) one of the most important abiotic stresses, it seemed highly relevant to us to also study the response of plants to drought stress in more detail. In literature, many drought stress experiments were performed under *in vitro* conditions, which do not reflect the stress situations that plants encounter under natural circumstances. Moreover, evaluation of drought stress tolerance of soil-grown plants is mainly focused on survival to long periods without watering. We first built a semi-automatic imaging and watering platform (WIWAM) in order to evaluate the growth of plants during controlled and mild drought conditions. The performance of this system was successfully tested and used for selecting transgenic lines for further analysis. The system will be further used to analyze the growth of transgenic lines, including WRKY15 overexpressing plants, during drought stress. We believe that it will be an important tool for future drought stress analysis and will certainly be very useful for others within the department.

Transcriptome analysis as efficient tool to elucidate the stress response of plants

Initially, transcriptome analysis were commonly used to study the response of plants to environmental stress. In addition, an increasingly amount of transcriptome data on transgenic plants with increased stress tolerance is now being produced to obtain more insight into the molecular network behind stress tolerance. Comparison of such stress-related transcriptome datasets showed that most regulators of stress tolerance work via independent mechanisms, which reflects the complexity of the response of plants to stress. Several important genes are induced by multiple stresses, but other stress-responsive genes are left unattended and it could be assumed that these are good candidates for the improvement of stress resistance in plants.

Of all *Arabidopsis* genes, GOLS2 is one of the most highly stress-responsive genes. GOLS2 expression is strongly induced by high temperatures, osmotic stress, salt, drought and the stress hormone ABA, and overexpression of GOLS2 renders plants more tolerant to drought stress. A microarray analysis was performed to get more insight into the mechanism of drought tolerance in GOLS2 overexpressor plants and this suggested that *myo*-inositol metabolism was altered in these plants. We are currently further exploring this interesting results because m*yo*-inositol is a central molecule in carbohydrate metabolism of plants, with various important functions during growth,

development and response to stress. Knock-out plants for different MIOX genes will be analyzed and crossed with GOLS2 overexpressor plants. Also other downstream genes were selected for further analysis. We are confident that our microarray analysis was a good approach to study the molecular mechanisms that underpin stress tolerance in GOLS2 transgenic plants, and that this will lead us to further study the defense response of plants to (drought) stress. It is also evident that future transcriptome analysis of plants with increased WRKY15 expression levels could contribute to elucidate the molecular mechanism downstream of WRKY15 and its functions during the stress response of plants.

Improving plant stress responses and yield at the same time: mission impossible?

Constitutive overexpression of stress-responsive genes in plants often leads to a negative effect on plant growth and development (yield penalty) under normal conditions. This seems logic, since stress-related genes are normally low expressed and only induced when the gene products are needed. Drought tolerant GOLS2 overexpressor plants are slightly smaller than untransformed plants. A role for *myo*-inositol could be expected as it possibly contributes to biomass determination in plants. During this thesis, we observed that also altering the expression levels of H_2O_2-induced genes can lead to changes in the phenotype of plants under normal conditions. Such genes are therefore interesting candidates to study the role for H_2O_2 during plant development in more detail. Interestingly, overexpression of WRKY15 had no negative, even a positive, effect on plant growth under control conditions. Kinematic analysis of GOLS2 and WRKY15 transgenic plants will be performed to study the observed phenotypes in more detail.

In conclusion, we believe that, by the analysis and comparison of transcriptome data, by the identification of H_2O_2-induced genes with putative important roles in plant development and stress defense, and by the development of tools to evaluate the stress response of plants, this work has delivered a valuable basis towards a better understanding of H_2O_2-signal transduction and the molecular mechanisms that control the defense response of plants to stresses. Especially the role of WRKY15 is worth following up. Its expression is induced by H_2O_2 and it encodes a transcription factor and could therefore be one of the central regulators of H_2O_2 signaling and plants defense response to stress. Overexpression of WRKY15 increased tolerance to oxidative stress, with a positive effect on plant growth under normal conditions. In addition, overexpression of WRKY15 altered the tolerance of plants to salt and osmotic stress.

CHAPTER 8

SUMMARY

Environmental conditions that limit the growth, development and yield of plants are divided into two types: (i) biotic stress, which is caused by interaction with other living organisms, and (ii) abiotic stress, which is defined as stress caused by non-living components of the environment and is associated with climate and soil factors. Plants must adapt to environmental stresses in order to survive. Common for all environmental stresses is that they induce the accumulation of harmful reactive oxygen species (ROS). However, ROS, such as hydrogen peroxide (H_2O_2), are now also considered to be important signal molecules that regulate the defense response of plants to stress (Foyer and Noctor, 2005). The stress response of plants is regulated at the transcriptional level by stress-inducible transcription factors (TFs) that control the expression of downstream defense genes. Many genes have been described that enhance stress tolerance when engineered in plants, but a major challenge for the future is to discover genes that confer broad-spectrum and long-lasting tolerance without affecting normal growth and development (**Chapter 1**).

In this thesis, different strategies were pursued to identify genes that are involved in the stress response of plants. In the first part of this work, we took advantage of available transcriptome data on the changes in gene expression caused by increased H_2O_2 levels. A genome-wide meta-analysis of H_2O_2-induced gene expression in *Synechocystis*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Arabidopsis thaliana* (*Arabidopsis)*, and the HeLa cell line from *Homo sapiens* was performed to assess the evolutionarily conservation of the transcriptional response to increased H_2O_2 levels (**Chapter 2**). Although a strong species-specific response was observed, we showed that the H_2O_2-induced gene expression in both yeast species (*S. cerevisiae* and *S. pombe*) was conserved and more similar to the response of *Arabidopsis* than to that of the HeLa cell line from *H. sapiens*. The H_2O_2-induced expression of evolutionarily conserved genes encoding DNAJ domain-containing heat shock proteins (HSPs), small GTP-binding proteins, calcium (Ca^{2+})-dependent protein kinases, and ubiquitin-conjugating enzymes was conserved across the eukaryotic kingdoms. It is known that these proteins function in fundamental biological processes, including the heat shock response (HSPs), cellular signaling (GTP-binding proteins and Ca^{2+}-dependent protein kinases) or ubiquitin-targeted protein degradation (ubiquitin-conjugating

enzymes), which have important functions during (oxidative) stress (Kregel *et al*., 2002; Finkel, 2001; Goldberg, 2003; Matozaki *et al*., 2000). Another observation was that the transcriptional induction of antioxidant genes, encoding enzymes that are involved in the protection against oxidative stress, was conserved in unicellular organisms, and this indicated that unicellular antioxidant systems are part of the oxidative stress-inducible adaptive responses. One of the goals of this comparative analysis was to identify genes (or families) with a conserved H_2O_2-induced expression and encoding proteins with unknowns functions. Ten families of unknown proteins were found and we suggest that these proteins are interesting candidates to study new aspects of H_2O_2 signaling.

Previous research in the research group resulted in an extensive collection of genes that are transcriptionally induced by H_2O_2 in *Nicotiana tabacum* (tobacco) or *Arabidopsis*, and this provided valuable data to study H_2O_2 signaling in plants (Vandenabeele *et al*., 2003; Vanderauwera *et al*., 2005). In this thesis, H_2O_2-induced genes were evaluated for a putative role during biotic and abiotic stress. H_2O_2-induced tobacco genes were knocked down in *Nicotiana benthamiana* and *Lycopersicon esculentum* (tomato) plants using virus-induced gene silencing (VIGS) and subsequently screened for involvement in the defense response against two necrotrophic pathogens, *Botrytis cinerea* and *Sclerotinia sclerotiorum* (**Chapter 3**). H_2O_2 and other ROS have a dual role during attack by necrotrophic pathogens. They can favor a necrotrophic infection by inducing cell death or they can increase resistance by timely induced expression of defense genes (van Kan, 2006; Asselbergh *et al*., 2007). Silencing of such defense genes via VIGS would then result in increased sensitivity. Our screen yielded four genes, encoding a mitogen-activated protein (MAP) kinase kinase kinase (NPK1), a MAP kinase kinase (NQK1), a heat shock protein (HSP) and a putative esterase / lipase protein, for which VIGS led to increased sensitivity to *Botrytis* and/or *Sclerotinia.* These genes might therefore be relevant candidates to increase the resistance of plants against necrotrophic pathogens. In addition, we showed that VIGS of four H_2O_2-induced genes, encoding a BYPASS protein, a DNA-directed RNA polymerase, a threonyl-tRNA synthetase and a proteasome 26S regulatory subunit, in *N. benthamiana* and tomato resulted in stunted plants with pleiotropic effects on normal plant development.

Our research group possesses a collection of transgenic *Arabidopsis* plants, mainly TFs, with perturbed expression levels of H_2O_2-induced genes. Several ROS-induced TFs have been described and some of these are involved in ROS signaling during abiotic stress (Mittler *et al*., 2004). *Arabidopsis* plants with increased expression levels of H_2O_2-induced genes were evaluated under H_2O_2-induced oxidative stress and heat stress to identify genes that enhance stress tolerance in plants (**Chapter 4**). We observed that several of the transgenic plants with increased levels of H_2O_2-

induced genes, including lines that overexpress the ATAF1 and WRKY33 TFs, were already negatively affected in their growth and development (yield penalty due to increased bleaching and growth retardation) when grown under normal conditions. It is possible that the phenotypic aberrations caused by overexpression of these TFs are due to altered expression of downstream genes of which the encoded proteins are involved in biological processes that control plant growth and development. The yield penalty of *Arabidopsis* plants with increased expression levels of H_2O_2-induced genes, together with our VIGS results in *N. Benthamiana* and tomato, thus indicate that H_2O_2, in addition to its role during plant stress responses, also is an important signal molecule during plant growth and development. Indeed, H_2O_2 was shown to affect cell growth, abscisic acid-induced stomatal closure and root gravitropism (Apel and Hirt, 2004). Disturbance of such H_2O_2-controlled processes in plants with altered levels of H_2O_2-induced genes can therefore be responsible for their phenotypic aberrations.

Evaluation of transgenic *Arabidopsis* lines with increased levels of H_2O_2-induced genes for altered tolerance to H_2O_2-induced oxidative stress and heat stress resulted in the identification of one TF, WRKY15, for which overexpression enhanced tolerance of plants to H_2O_2. In addition, overexpression of WRKY15 also increased resistance to osmotic stress, as well as sensitivity to salt stress. We hypothesize that WRKY15 acts in a H_2O_2-activated MAPK cascade that is part of the oxidative stress signal transduction network during the stress response of *Arabidopsis*.

Among all environmental stresses, drought is one of the greatest global constraints for agriculture. In **chapter 5**, we studied the growth of *Arabidopsis* under drought stress conditions. To control soil water concentrations in soil-grown plants, a semi-automated platform, called WIWAM (weighing, imaging and watering machine), was designed. WIWAM offers a platform to study routinely the growth of plants under controlled watering conditions. For example, it can be used to study short-term growth adaptation to small differences in soil water concentration and to analyze the long-term growth response of plants to constant (mild) drought stress conditions, as well as growth responses to gradual soil-drying. WIWAM was evaluated by analyzing the growth of wild-type (WT) *Arabidopsis* plants, and stress-tolerant transgenic plants that ectopically express AVP1 ($AVP1^{OE}$) encoding a vacuolar H^+-pyrophosphatase, and GOLS2 ($GOLS2^{OE}$) encoding an enzyme involved in galactinol biosynthesis. Our results showed that drought indeed negatively affected the growth and yield of plants, and indicated that $AVP1^{OE}$ and $GOLS2^{OE}$ plants have different growth rates compared to WT plants. The reduced growth rate of $GOLS2^{OE}$ plants resulted in a yield penalty caused by a reduced rosette area.

To further study the molecular mechanisms underpinning plant responses to drought stress, a microarray analysis of *Arabidopsis* plants overexpressing GOLS2 was performed (**Chapter 6**). GOLS2

catalyzes the production of galactinol, a sugar that protects cellular structures against dehydration, by using *myo*-inositol (MI) and sucrose as substrates. Hence, transgenic plants that overexpress GOLS2 produce more galactinol (and other related molecules such as raffinose) and are more tolerant to drought stress (Taji *et al*., 2002). The microarray data indicated that the expression of genes of which the products are involved in carbohydrate metabolism were affected in $GOLS2^{OE}$ plants. The expression of MIOX2, encoding a key enzyme in the MI oxygenation pathway, was most strongly induced by GOLS2 overexpression. The MI oxygenation pathway is an alternative route for the production of cell wall precursors. This result indicates that ectopic expression of GOLS2 does not only alter the carbon partitioning between sucrose and raffinose, but also redirects MI metabolism towards cell wall biosynthesis. An interesting questions that still remains is how these processes relate to the increased drought tolerance of $GOLS2^{OE}$ plants and what are the mechanisms that cause the negative effect on the yield of these plants.

In conclusion, this thesis has led to the discovery of plant genes for which the encoded proteins are involved in growth, development and yield of plants, or the defense response to biotic or abiotic stress, and this was achieved by using different approaches and technologies. Further research will have to reveal if this knowledge can be used for the improvement of crops and other economically important plants.

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SAMENVATTING

Omgevingsfactoren die de groei, ontwikkeling en opbrengst van planten negatief beïnvloeden kunnen ingedeeld worden in twee types: biotische stress, die veroorzaakt wordt door interactie met andere levende organismen, en abiotische stress veroorzaakt door niet-levende componenten uit de omgeving, en deze zijn vooral geassocieerd met klimaatwijzigingen en grond- en luchtvervuiling. Planten moeten zich aanpassen aan stress uit de omgeving om te kunnen overleven. Een centraal gebeuren tijdens stress in planten is de accumulatie van schadelijke vrije zuurstofradicalen (VZR), maar VZR, waaronder waterstofperoxide (H_2O_2), worden nu ook beschouwd als belangrijke signaalmoleculen in defensieresponse tegen stress. De defensiemechanismen van planten wordt voornamelijk gecontroleerd door stressgeïnduceerde transcriptiefactoren (TFen) die de expressie controleren van genen die betrokken zijn in bescherming van de plant. Ondertussen zijn reeds honderden genen beschreven die de stresstolerantie van planten kunnen verhogen wanneer hun expressieniveaus aangepast worden, maar de uitdaging voor de toekomst is om genen te ontdekken waarvan de genproducten (eiwitten) de tolerantie verhogen tegen een brede waaier aan verschillende stress factoren en dit voor een langdurige tijd, zonder een negatief effect te veroorzaken op de normale groei en ontwikkeling van planten (**Hoofdstuk 1**).

In deze thesis werden verschillende strategieën gevolgd om nieuwe genen te vinden waarvan de geëncodeerde eiwitten betrokken zijn bij de stress response van planten. In het eerste deel van de thesis hebben we gebruik gemaakt van beschikbare transcriptoom data over veranderingen in genexpressie die optreden ten gevolge van H_2O_2 accumulatie. In een eerste benadering hebben we een genoomwijde analyse uitgevoerd van de H_2O_2-geïnduceerde genexpressie in de bacterie *Synechocystis* en vier eukaryoten, waarvan twee gisten (*Saccharomyces cerevisiae* en *Schizosaccharomyces pombe*), de landplant *Arabidopsis thaliana* (*Arabidopsis*), en een cellijn (HeLa) afkomstig van *Homo sapiens*, om de evolutionaire conservering van de transcriptionele response op verhoogde H_2O_2 concentraties te bestuderen (**Hoofdstuk 2**). Alhoewel een sterke species-specifieke response werd vastgesteld, konden we aantonen dat de H_2O_2-geïnduceerde response sterk geconserveerd was in de twee gisten, en dat deze meer gelijkenissen vertoonden met *Arabidopsis* dan met de HeLa cellijn afkomstig van mens. Vier genfamilies, coderend voor hitte shock proteïnen met een DNAJ domain (HSPs), kleine GTP-bindende proteïnen, calcium-afhankelijke proteïne kinasen, en ubiquitin-conjugerende enzymes zijn geconserveerd binnen eukaryote species en vertoonden ook een geconserveerde H_2O_2-geïnduceerde genexpressie over de eukaryote rijken.

Deze proteïnen zijn betrokken in fundamentele biologische processen, zoals de response op hoge temperaturen (HSPs), cellulaire signaaltransductie (kleine GTP-bindende proteïnen en calcium-afhankelijke protein kinasen) of ubiquitin-gecontroleerde proteïne degradatie (ubiquitin-conjugerende enzymes), en hebben belangrijke functies uit tijdens stress (Kregel *et al*., 2002; Finkel, 2001; Goldberg, 2003; Matozaki *et al*., 2000). Een andere belangrijke observatie was dat de inductie van genen die coderen voor antioxidantia, en dus betrokken zijn in de bescherming tegen oxidatieve stress, enkel gebeurt in de unicellulaire organismen (*Synechocystis* en de beide gisten), wat erop wijst dat de unicellulaire antioxidant system systemen deel uit maken van een oxidatieve stress-geïnduceerde adaptieve response. Een van de doelen van deze vergelijkende analyse was om genen (of families van genen) met een geconserveerde H_2O_2-geïnduceerde expressie te identificeren en waarvan de geëncodeerde eiwitten nog ongekende functies hebben. Tien families werden gevonden en de genen daarin zijn mogelijks interessante kandidaten voor het bestuderen van nieuwe aspecten van stress signaaltransductie.

Voorafgaand onderzoek in de onderzoeksgroep resulteerde in een uitgebreide collectie van genen die geïnduceerd zijn door H_2O_2 in *Nicotiana tabacum* (tabak) of *Arabidopsis*, en die genen zijn waardevolle kandidaten voor de studie van H_2O_2-signaaltransductie in planten (Vandenabeele *et al*., 2003; Vanderauwera *et al*., 2005). In deze thesis werden H_2O_2-geïnduceerde genen geëvalueerd voor een mogelijke rol tijdens biotische en abiotische stress. In **hoofdstuk 3** werd beschreven hoe H_2O_2-geïnduceerde tabaksgenen uitgeschakeld werden in *Nicotiana benthamiana* en *Lycopersicon esculentum* (tomaat) via virus-geïnduceerde genuitdoving (VIGS) om hun betrokkenheid na te gaan tijdens de defensieresponse van planten tegen twee necrotrofe pathogenen, *Botrytis cinerea* and *Sclerotinia sclerotiorum*. H_2O_2 (en andere VZR) hebben een dubbele rol tijdens infectie door necrotrofe pathogenen: enerzijds kunnen ze een infectie bevorderen door het afdoden van plantcellen, maar anderzijds kunnen ze ook een specifiek defensieresponse opwekken, bvb induceren van defensie genen, die leidt tot resistentie (Asselbergh *et al*., 2007). Uitdoving van zulke defensiegenen via VIGS zou dan resulteren in verhoogde gevoeligheid. Vier genen, coderend voor een mitogen-activated protein (MAP) kinase kinase kinase (NPK1), een MAP kinase kinase (NQK1), een HSP en een eiwit met mogelijke esterase of lipase activiteiten, werden gevonden waarvan genuitdoving leidde tot verhoogde gevoeligheid van *N. benthamiana* en tomaat tegen *Botrytis* and/or *Sclerotinia.* Deze genen kunnen daarvoor relevante kandidaten zijn om de resistentie van planten tegen necrotrofe pathogenen te verhogen. Daarnaast werden aangetoond dat VIGS van vier H_2O_2-geïnduceerde tabaksgenen, coderend voor een BYPASS protein, een DNA-afhankelijk RNA polymerase, een aminozuur-tRNA synthetase en een

proteasome 26S regulatorische subeenheid, resulteerde in compacte planten met pleiotrope effecten op hun normale groei en ontwikkeling.

Ons labo beschikt ook over een collectie transgene *Arabidopsis* planten met gewijzigde expressieniveaus van H_2O_2-geïnduceerde genen. Er zijn in de literatuur reeds verschillende TFen beschreven waarvan de genexpressie geïnduceerd wordt door VZR, én die betrokken in de defensieresponse van planten tegen stress (Mittler *et al*., 2004). Transgene *Arabidopsis* planten met verhoogde expressieniveaus van H_2O_2-geïnduceerde genen, hoofdzakelijk TFen, werden geëvalueerd voor gewijzigde tolerantie tegen abiotische om nieuwe genen te identificeren die de defensie response van planten tegen abiotische stress controleren (**Hoofdstuk 4)**. Verschillende van deze transgene planten, waaronder planten die de ATAF1 en WRKY33 TFen tot overexpressie brengen, vertoonden echter al ernstige fenotypische gebreken (verbleking en groeivertraging) wanneer ze gegroeid werden onder normale omstandigheden. Dit is mogelijks verzoorzaakt doordat overexpressie van TFen kan leiden to een gewijzigde expressie van doelgenen die coderen voor eiwitten met een belangrijke rol in fundamentele biologische processen. Deze resultaten, tesamen met de observaties in *N. benthamiana* en tomaat, suggereren dat H_2O_2, naast een rol tijdens de stress response, ook een belangrijke signaalfunctie heeft tijdens groei en ontwikkeling van planten. Het is inderdaad al bekend dat H_2O_2 celgroei, sluiting van de huidmondjes en wortel gravitropisme controleert (Apel and Hirt, 2004). Verstoring van zulke H_2O_2-gecontroleerde processen kan dus verantwoordelijk zijn voor de fenotypische abnormaliteiten in de groei en ontwikkeling van planten met gewijzigde expressieniveaus van H_2O_2-geïnduceerde genen.

Evaluatie van de transgene planten tijdens hitte stress en verhoogde H_2O_2 concentraties toonde aan dat planten met verhoogde expressieniveaus van de WRKY15 TF meer tolerant waren tegen H_2O_2-geïnduceerde oxidatieve stress. Onze hypothese is dat WRKY15 een doeleiwit is van een H_2O_2-geactiveerde MAP kinase cascade die mogelijks een belangrijke onderdeel is van het oxidatieve stress signalisatienetwerk van *Arabidopsis*. Het verhogen van WRKY15 expressieniveaus resulteerde ook in verhoogde resistentie tegen osmotische stress en verhoogde gevoeligheid tegen zoutstress.

Van alle stressfactoren is droogte een van de belangrijkste problemen voor de land- en tuinbouw. Daarom werd in **hoofdstuk 5** de groei van *Arabidopsis* tijdens gelimiteerde waterbeschikbaarheid bestudeerd. Omdat het controleren van de waterconcentraties hiervoor noodzakelijk is werd een semiautomatisch systeem op punt gesteld. Dit systeem, genoemd WIWAM (weighing, imaging and watering machine), biedt een platform om routinematig de groei van planten tijdens gecontroleerde (droogte) condities te bestuderen. WIWAM zou bijvoorbeeld gebruikt kunnen worden om de groei aanpassing van planten ten gevolge van kleine wijzigingen in

waterconcentraties op korte termijn te bestuderen, maar eveneens om wijzingen in groei ten gevolge van een langdurige bloostelling aan constante (milde) droogte condities en om het effect van een graduele uitdroging van de grond op de groei te analyseren. WIWAM werd gebruikt om de groei te analyseren van wild type (WT) *Arabidopsis* planten, en stress-tolerante transgene planten met verhoogde expressieniveaus van AVP1 ($AVP1^{OE}$), coderend voor een H^+ pyrophosphatase in de vacuole, en transgene planten met verhoogde expressieniveaus van GOLS2 ($GOLS2^{OE}$), coderend voor een enzyme verantwoordelijk voor de biosynthese van galactinol. Onze resultaten toonden aan dat droogte inderdaad een negatief effect heeft op de groei en opbrengst van planten en dat $AVP1^{OE}$ en $GOLS2^{OE}$ planten een verschillende groeisnelheid hebben dan WT planten. De gereduceerde groeisnelheid van $GOLS2^{OE}$ planten resulteerde in een verminderde opbrengst. Om de moleculaire mechanismen te bestuderen die verantwoordelijk zijn voor de droogtetolerantie van *Arabidopsis* werden genoomwijde microarray analyses uitgevoerd op $GOLS2^{OE}$ planten, wat toelaat om de expressie van alle genen in het genoom tezamen te bestuderen (**Hoofdstuk 6**). GOLS2 is een enzyme dat de synthese van galactinol (een suiker die celstructuren beschermt tegen droogte) katalyseert vertrekkende van *myo*-inositol (MI) en sucrose als substraten. $GOLS2^{OE}$ transgene planten produceren meer galactinol (en verwante moleculen zoals raffinose) en vertonen daarom een verhoogde droogtetolerantie (Taji *et al*., 2002). De microarray data van $GOLS2^{OE}$ planten toonde gewijzigde expressie aan van genen waarvan de producten betrokken zijn in carbohydraat metabolisme, voornamelijk dit van MI. De expressie van MIOX2, coderend voor een belangrijk enzyme in MI oxygenatie, was het sterkst geïnduceerd. MI oxygenatie is een alternatieve syntheseweg voor de productie van celwand precursoren. Dit resultaat toont aan dat verhogen van GOLS2 expressie niet enkel de koolstofverdeling tussen sucrose en raffinose wijzigt, maar ook het MI metabolisme dirigeert in de richting van celwand biosynthese. Een belangrijke vraag die rest is hoe het gewijzigde MI metabolisme gerelateerd is aan droogtetolerantie en opbrengst van $GOLS2^{OE}$ planten.

Samengevat kan gesteld worden dat deze thesis heeft geleid tot de identificatie van genen waarvan de geëncodeerde eiwitten betrokken zijn bij de groei, ontwikkeling en opbrengst van planten, en van genen waarvan de eiwitten mogelijks betrokken zijn bij de stress response van planten, en dit door middel van diverse strategieën en verschillende technologieën. Verder onderzoek zal moeten uitmaken of deze kennis kan gebruikt worden voor opbrengstverbetering in economisch relevante gewassen.

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PART V

ADDENDUM

Supplementary Table S1. *Arabidopsis* stress tolerance genes

Gene	AGI	Molecular Function	Species	D	S	O	H	C/F	Ox	M	Construct	Reference
Detoxification and redox control												
PCS1	AT5G44070	Phytochelatin synthesis	Rapeseed							1	gain-of-function	Gasic and Korban, 2007
			Arabidopsis							1	gain-of-function	Lee *et al.*, 2003
			Arabidopsis							1	gain-of-function	Li *et al.*, 2004
			Tobacco							1	gain-of-function	Pomponi *et al.* 2006
AAO	AT5G21100	Ascorbate oxidase	Tobacco		1				1		loss-of-function	Yamamoto *et al.*, 2005
			Arabidopsis		1				1		loss-of-function	Yamamoto *et al.*, 2005
ALDH3I3	AT4G34240	Aldehyde dehydrogenase	*Arabidopsis*	1	1				1	1	gain-of-function	Sunkar *et al.* 2003
			Arabidopsis	1	1						gain-of-function	Kotchoni *et al.* 2006
ALDH7B4	AT1G54100	Aldehyde dehydrogenase	*Arabidopsis*	1	1						gain-of-function	Kotchoni *et al.* 2006
AOX1	AT3G22370	Alternative oxidase	*Arabidopsis*					1			gain-of-function	Fiorani *et al.* 2005
APX1	AT1G07890	Ascorbate peroxidase	*Arabidopsis*		1	1	1	1	-1		loss-of-function	Miller *et al.*, 2007
APX3	AT4G35000	Ascorbate peroxidase	Tobacco						1		gain-of-function	Wang *et al.* 1999
			Tobacco	1							gain-of-function	Juqiang Yan *et al.* 2003
FRO2	AT1G01580	Ferric chelate reductase responsible	*Arabidopsis*							1	gain-of-function	Connolly *et al.*, 2003
GI-3	AT1G22770	gigantea	*Arabidopsis*						1		loss-of-function	Kurepa *et al.*, 1998
GPX3	AT2G43350	Glutathione peroxidase	*Arabidopsis*	1		1					gain-of-function	Miao *et al.*, 2006
tAPX	AT1G77490	t-ascorbate peroxidase	*Arabidopsis*		1	1	1				loss-of-function	Miller *et al.*, 2007
			Arabidopsis						1		gain-of-function	Murgia *et al.*, 2004
MDAR1	AT3G52880	Peroxisomal monodehydroascorbate reductase	Tobacco		1	1					gain-of-function	Eltayeb *et al.*, 2007
MT2a	AT3G09390	Metallothionein	*Vicia faba*							1	gain-of-function	Lee *et al.*, 2004
MT3	AT3G15353	Metallothionein	*Vicia faba*							1	gain-of-function	Lee *et al.*, 2004
NAS	??	Nicotianamine synthase	Tobacco							1	gain-of-function	Douchkov *et al.*, 2005
PCS1	AT5G44070	Phytochelatin synthesis	Rapeseed							1	gain-of-function	Gasic and Korban, 2007
RCI3	AT1G05260	Rare Cold Inducible gene 3, encodes peroxidase	*Arabidopsis*	1	1						gain-of-function	Llorente *et al.*, 2002
SOD	AT3G56350	Mn superoxide dismutase	*Arabidopsis*		1						gain-of-function	Wang *et al.*, 2004
VTC1	AT2G39770	Vitamin C defective 1, encodes mannose-1-pyrophosphatase	*Arabidopsis*				1				loss-of-function	Larkindale *et al.*, 2005
			Arabidopsis		1						loss-of-function	Huang *et al.*, 2005
VTC2	AT4G26850	Vitamin C defective 2, encodes mannose-1-pyrophosphatase	*Arabidopsis*				1				loss-of-function	Larkindale *et al.*, 2005
SOD/CSD2	AT2G28190	miRNA resistanct form of chloroplastic Cu/Zn superoxide dismutases	*Arabidopsis*						1	1	gain-of-function	Sunkar *et al.*, 2006
SOD	AT4G25100	Fe superoxide dismutase	Tobacco						1		gain-of-function	Van Camp *et al.* 1996
APX1/tAPX	AT1G07890 and AT1G77490	Ascorbate peroxidase	*Arabidopsis*		1		1	-1			loss-of-function	Miller *et al.*, 2007
DNA repair/replication												
PARP1	AT2G31320	Poly(ADP-ribose) polymerase	Rapeseed	1			1		1		loss-of-function	Block *et al.*, 2005
			Arabidopsis	1			1		1		loss-of-function	Block *et al.*, 2005
PARP2	AT4G02390	Poly(ADP-ribose) polymerase	Rapeseed	1			1		1		loss-of-function	Block *et al.*, 2005
			Arabidopsis	1			1		1		loss-of-function	Block *et al.*, 2005
UVH6	AT1G03190	UV-sensitive mutant	*Arabidopsis*				1				loss-of-function	Larkindale *et al.*, 2005
UVI1	unmapped	Unmapped mutant	*Arabidopsis*								loss-of-function	Tanaka *et al.*, 2002
DHAR	AT1G19570?	Dehydroascorbate reductase	Tobacco	1	1	1					gain-of-function	Elsadig *et al.* 2006
FAD7	AT3G11170	Fatty acid desaturation	Tobacco					1			gain-of-function	Khodakovskaya *et al.* 2006
FAD8	AT5G05580	Fatty acid desaturation	Tobacco	1		1	-1				gain-of-function	Zhang *et al.*, 2005
GPAT	?	Glycerol-3-phosphate acyltransferase of chloroplasts	Rice					1			gain-of-function	Ariizumi *et al.*, 2002
PLDalfa1	AT3G15730	Phospholipase Alfa, modulation of COR genes	*Arabidopsis*					1			loss-of-function	Rajashekar *et al.*, 2006
PLDdelta	AT4G35790	Phospholipase Delta	*Arabidopsis*					1			gain-of-function	Li *et al.*, 2004
LTL1	AT3G04290	GDSL-type lipase	*Arabidopsis*		1						gain-of-function	Naranjo *et al.*, 2006
Hormone Biosynthesis												
ABA2/GIN	AT1G52340	Cytosolic short-chain dehydrogenase/reductase	*Arabidopsis*		1						gain-of-function	Lin *et al.*, 2007
CYP707A3	AT5G45340	ABA 8'-hydroxylase activity	*Arabidopsis*	1							loss-of-function	Umezawa *et al.* 2006
IPT	??	Isopententyltransferase	*Arabidopsis*								gain-of-function	Nguyen Huynh *et al.*, 2005
NCED3/STO1	AT3G14440	Salt Tolerant 1, protein binds to a Myb transcription factor	*Arabidopsis*		1						gain-of-function	Shuuichi and Takano 2003
			Arabidopsis		1	1					loss-of-function	Ruggiero *et al.*, 2004
			Arabidopsis	1							gain-of-function	Iuchi *et al.*, 2001

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Molecular protection												
COR15a	AT2G42540	LEA	*Arabidopsis*					1			gain-of-function	Steponkus *et al.* 1998
DHN (LTI29/ERD10+LTI30)	AT1G20450 and AT3G50970	Dehydrin	*Arabidopsis*					1			gain-of-function	Puhakainen *et al.*, 2004
DHN (RAB18+COR47)	AT5G66400 and AT1G20440	Dehydrin	*Arabidopsis*					1			gain-of-function	Puhakainen *et al.*, 2004
ERD15	AT2G41430	Early responsive to dehydratation	*Arabidopsis*	1				1			loss-of-function	Kariola *et al.*, 2006
F9E10.5	AT1G75100	auxilin-like gene	*Arabidopsis*							1	gain-of-function	Ezaki *et al.*, 2007
HSP101	AT1G74310	Heat shock protein	Rice				1				gain-of-function	Katiyar-Agarwal *et al.* 2003
			Arabidopsis				1				gain-of-function	Queitsch *et al.* 2000
HSP17.6A	AT5G12030	Heat shock protein	*Arabidopsis*	1	1						gain-of-function	Sun *et al.*, 2001
LEA5	AT4G02380	Late embryogenesis abundant	*Arabidopsis*	-1					1		gain-of-function	Mowla *et al.*, 2006
DJA2	AT5G22060	DNAj domain containing molecular chaperones	*Arabidopsis*				1				gain-of-function	Li *et al.*, 2007
DJA3	AT3G44110	DNAj domain containing molecular chaperones	*Arabidopsis*				1				gain-of-function	Li *et al.*, 2007
GOLS2	AT1G56600	Galactinol and raffinose accumulation	*Arabidopsis*	1							gain-of-function	Taji *et al.* 2002
LWR1	unmapped	Solute accumulation (proline)	*Arabidopsis*			1					loss-of-function	Verslues and Bray, 2004
P5CR	AT5G14800	Pyrroline carboxylate reductase (proline)	Soybean	1			1				gain-of-function	Kocsy *et al.*, 2005
			Soybean	1							gain-of-function	De Ronde *et al.*, 2004
			Soybean		1		1				gain-of-function	De Ronde *et al.* 2001
P5CS	AT2G39800?	Pyrroline carboxylate synthase (proline synthesis)	Potato		1						gain-of-function	Hmida-Sayari *et al.*, 2005
			Tobacco					1			gain-of-function	Parvanova *et al.*, 2004
			Tobacco					1			gain-of-function	Parvanova *et al.*, 2004
			Petunia	1							gain-of-function	Yamada *et al.*, 2005
ProDH	AT3G30775	Proline dehydrogenase	*Arabidopsis*		1			1			loss-of-function	Nanjo *et al.*, 1999
TPS1	AT1G78580	Trehalose-6-phosphate synthase	*Arabidopsis*	1							gain-of-function	Avonce *et al.*, 2004
			Tobacco	1							gain-of-function	Almeida *et al.*, 2007
OAT		Ornithine-delta-aminotransferase	Rice	1	1						gain-of-function	Wu *et al.*, 2005
Post-transcriptional control												
DHS	AT5G05920	Deoxyhypusine synthase, eIF5a activation	*Arabidopsis*	1							loss-of-function	Wang *et al.*, 2003
LOS4/CRYOPHITE	AT3G53110	DEAD-Box RNA Helicase and has RNA-dependent ATPase activity	*Arabidopsis*				-1	1			loss-of-function	Gong *et al.*, 2005
RZ-1a	AT3G26420	RNA chaperone protein	*Arabidopsis*	1	1						loss-of-function	Kim *et al.*, 2007
			Arabidopsis					1			gain-of-function	Kim *et al.*, 2005
SR-like	AT5G37370	Splicing protein	*Arabidopsis*		1						gain-of-function	Forment *et al.*, 2002
STRS1	AT1G31970	Stress Response Suppressor 1, DEAD-box RNA helicase	*Arabidopsis*		1	1	1				loss-of-function	Kant *et al.*, 2007
STRS2	AT5G08620	Stress Response Suppressor 2, DEAD-box RNA helicase	*Arabidopsis*		1	1	1				loss-of-function	Kant *et al.*, 2007
GRP2	AT4G13850	Glycine-rich RNA binding protein	*Arabidopsis*		1			1			gain-of-function	Kim *et al.*,2007
FTA	AT5G40280	Farnesyltransferase	*Arabidopsis*	1							loss-of-function	Wang *et al.*, 2005
FTB/ERA1	AT5G40280	Farnesyltransferase	*Arabidopsis*	1							loss-of-function	Pei *et al.*, 1998
			Arabidopsis	1							loss-of-function	Wang *et al.*, 2005
			Rapeseed	1							loss-of-function	Wang *et al.*, 2005
SDIR1	AT3G55530	Salt and Drought-Inducible RING finger E3 ligase	*Arabidopsis*	1	-1						gain-of-function	Zhang *et al.*, 2007
ORE9	AT2G42620	the F-box leucine-rich repeat family	*Arabidopsis*						1		loss-of-function	Woo *et al.*, 2004
PMSR4	AT4G25130	Peptide methionine sulfoxide reductase	*Arabidopsis*						1		gain-of-function	Romero *et al.*, 2004
Signaling												
ABI1+HAB1	AT4G26080 and AT1G72770	Genes Involved in ABA signal transduction.	*Arabidopsis*	1							loss-of-function	Saez *et al.*, 2006
GSK1	AT1G06390	Homologue of GSK3/shaggy-like protein kinase	*Arabidopsis*	1	1						gain-of-function	Piao *et al.*, 2001
AHK1/ATHK1	AT2G17820	Cytokinin receptor histidine kinase	*Arabidopsis*	1							gain-of-function	tran *et al.*, 2007
MKK2	AT4G29810	MAPKK	*Arabidopsis*		1			1			gain-of-function	Teige *et al.*, 2004
MKK9	AT1G73500	MAPKKK	*Arabidopsis*		1	1					loss-of-function	Alzwiy *et al.*, 2007
NDPK2	AT5G63310	NDP kinases	*Arabidopsis*		1			1	1		gain-of-function	Moon *et al.*, 2003
			Potato				1		1		gain-of-function	Tang *et al.*, 2007
PP2CA	AT3G11410	Protein phosphatase 2C	*Arabidopsis*					1			loss-of-function	Tahtiharju and Palva, 2001
RAB7	AT1G49300	Small GTPase, RAB family	*Arabidopsis*		1	1					gain-of-function	Mazel *et al.*, 2004
RGS1	AT3G26090	Regulation of G-protein signalling	*Arabidopsis*	1							gain-of-function	Chen *et al.* 2006
SRK2C	AT1G78290	Protein kinase	*Arabidopsis*	1		1					gain-of-function	Umezawa *et al.*, 2004
PP7	AT5G63870	Calmodulin-binding protein phosphatase PP7	*Arabidopsis*				1				gain-of-function	Liu *et al.*, 2007
AHK2	AT5G35750	Cytokinin receptor histidine kinase	*Arabidopsis*	1	1						loss-of-function	Tran *et al.*, 2007
AHK3	AT1G27320	Cytokinin receptor histidine kinase	*Arabidopsis*	1	1						loss-of-function	Tran *et al.*, 2007

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Signaling (continued)												
TOR	AT1G50030	Target of rapamycin	*Arabidopsis*		1						gain-of-function	Deprost *et al.*, 2007
CBL1	AT4G17615	Calcineurin B-like Calcium Sensor Proteins	*Arabidopsis*	-1	-1			1			loss-of-function	Cheong *et al.* 2003
			Arabidopsis	1							gain-of-function	Albrecht *et al.*, 2003
			Arabidopsis	1	1			-1			gain-of-function	Cheong *et al.* 2003
CAMBP25	AT2G41010	Calmodulin (CaM)-binding protein	*Arabidopsis*		1	1					loss-of-function	Perruc *et al.*, 2004
CIPK23	AT1G30270	CBL-interacting protein kinase	*Arabidopsis*	1							loss-of-function	Cheong *et al.*, 2007
CPK23	AT4G04740	Calcium-dependent protein kinase	*Arabidopsis*	1	1						loss-of-function	Ma and Wu, 2007
CBL1CBL9		Calcineurin-B-like protein	*Arabidopsis*	1							loss-of-function	Cheong *et al.*, 2007
EIN2/ORE3	AT5G03280	ethylene mutant	*Arabidopsis*						1		loss-of-function	Woo *et al.*, 2004
CTR1	AT5G03730	Serine/threonine/tyrosine kinase (Constitutive Transcriptional Response)	*Arabidopsis*		1						loss-of-function	Achard *et al.*, 2006
GLI1	AT1G80460	Glycerol kinase	*Arabidopsis*	1	1	1		1	1		loss-of-function	Eastmond, 2004
Transcription												
ABF2/AREB1	AT1G45249	ABA RE binding factor	*Arabidopsis*	1							gain-of-function	Fujita *et al.* 2005
			Arabidopsis	1	1		1		1		gain-of-function	Kim *et al.*, 2005
ABF3	AT4G34000	ABA RE binding factor	*Arabidopsis*	1	-1						gain-of-function	Kang *et al.*, 2002
			Arabidopsis	-1	1						loss-of-function	Kim *et al.*, 2005
			Rice	1							gain-of-function	Oh *et al.*, 2005
ABF4	AT3G19290	ABA RE binding factor	*Arabidopsis*	-1	1						loss-of-function	Kim *et al.*, 2005
			Arabidopsis	1	-1						gain-of-function	Kang *et al.*, 2002
ABI3	AT3G24650	Transcription factor	*Arabidopsis*					1			gain-of-function	Tamminen *et al.* 2001
ABI3-14	AT3G24650	Transcription factor	*Arabidopsis*				1				loss-of-function	Tamura *et al.*, 2006
ABO1/ELO2	AT5G13680	Transcription Elongator complex subunit	*Arabidopsis*	1					1		loss-of-function	Chen *et al.*, 2006
ANAC002	AT1G01720	Transcription Factor with NAC domain	*Arabidopsis*	1							loss-of-function	Lu *et al.*, 2007
ANAC019	AT1G52890	Transcription Factor with NAC domain	*Arabidopsis*	1							gain-of-function	Tran *et al.*, 2004
ANAC055/NAC3	AT3G15500	Transcription Factor with NAC domain	*Arabidopsis*	1							gain-of-function	Tran *et al.*, 2004
ANAC072/RD26	AT4G27410	Transcription Factor with NAC domain	*Arabidopsis*	1							gain-of-function	Tran *et al.*, 2004
CBF1 / DREB1B	AT4G25490	Transcription Factor (Cold binding factor, Drought-Responsive Element Binding protein)	Potato					1			gain-of-function	Pino *et al.*, 2007
			Poplar					1			gain-of-function	Benedict *et al.* 2006
			Tomato					1			gain-of-function	Hsieh *et al.* 2002
			Arabidopsis					1			gain-of-function	Jaglo-Ottosen *et al.* 1998
			Arabidopsis					1			gain-of-function	Gilmour *et al.* 2004
CBF2 / DREB1C	AT4G25470	Transcription Factor (Cold binding factor, Drought-Responsive Element Binding protein)	*Arabidopsis*	1	1			1			loss-of-function	Novillo *et al.*, 2004
			Arabidopsis					1			gain-of-function	Gilmour *et al.* 2004
CBF3 / DREB1A	AT4G25480	Transcription Factor (Cold binding factor, Drought-Responsive Element Binding protein)	Rice	1	1			1			gain-of-function	Oh *et al.*, 2005
			Wheat	1							gain-of-function	Pellegrineschi *et al.*, 2004
			Tobacco	1				1			gain-of-function	Kasuga *et al.*, 2004
			Arabidopsis	1	1			1			gain-of-function	Kasuga *et al.* 1999
			Potato					1			gain-of-function	Pino *et al.*, 2007
CBF4/DREB1D	AT5G51990	Transcription Factor (Cold binding factor, Drought-Responsive Element Binding protein)	*Arabidopsis*	1				1			gain-of-function	Haake *et al.*, 2002
CPL1/FRY2	AT4G21670	Transcriptional repressor, C-terminal phosphatase-like	*Arabidopsis*		1			-1			loss-of-function	Xiong *et al.*, 2002
DREB2A	AT5G05410	Transcription Factor (Drought-Responsive Element Binding protein)	*Arabidopsis*				1				gain-of-function	Sakuma *et al.* 2006
			Arabidopsis	1				1			gain-of-function	Sakuma *et al.* 2006
HD2C	AT5G03740	Histone deacetylase	*Arabidopsis*	1	1						gain-of-function	Sridha and Wu, 2006
HSF1	AT4G17750	Transcription Factor, Heat shock factor 1	*Arabidopsis*				1				gain-of-function	Lee *et al.* 1995
HSF3	AT5G16820	Transcription Factor, Heat shock factor 1	*Arabidopsis*				1				gain-of-function	Prändl *et al.*, 1998
HSFA2	AT2G26150	Transcription Factor, Heat shock factor 1	*Arabidopsis*				1		1		gain-of-function	Li *et al.*, 2005
			Arabidopsis								gain-of-function	Ayako *et al.*, 2006
			Arabidopsis		1	1	1				gain-of-function	Ogawa *et al.*, 2007
ICE1	AT3G26744	Transcription Factor	*Arabidopsis*					1			gain-of-function	Chinnusamy *et al.*, 2003
MBF1a	AT2G42680	Multiprotein bridging factor 1a	*Arabidopsis*		1						gain-of-function	Kim *et al.*, 2007
MBF1c	AT3G24500	Multiprotein bridging factor 1c	*Arabidopsis*		1	1	1				gain-of-function	Suzuki *et al.*, 2005

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Transcription (continued)												
MYB2+MYC2	AT2G47190 (MYB2) and AT1G32640 (MYC2)	Transcription factors	*Arabidopsis*			1					gain-of-function	Abe *et al.*, 2003
MYB60	AT1G08810	Transcription factor	*Arabidopsis*	1							loss-of-function	Cominelli *et al.*, 2005
SZF1	??	Transcription factor CCCH-type zinc finger proteins	*Arabidopsis*		1						gain-of-function	Sun *et al.*, 2007
SZF2	??	Transcription factor CCCH-type zinc finger proteins	*Arabidopsis*		1						gain-of-function	Sun *et al.*, 2007
SHN1	AT1G15360	Transcription factor Shine-clan AP2	*Arabidopsis*	1							gain-of-function	Aharoni *et al.*, 2004
STZ/ ZAT10	AT1G27730	Transcription Factor, Cys2/His2-Type Zinc-Finger Proteins	*Arabidopsis*		1	1	1				gain-of-function	Mittler *et al.*, 2006
			Arabidopsis		1	1	1				loss-of-function	Mittler *et al.*, 2006
			Arabidopsis	1							gain-of-function	Sakamoto *et al.*, 2004
XERICO	AT2G04240	Transcription factor, RING-H2 zinc finger	*Arabidopsis*	1	-1	-1					gain-of-function	Ko *et al.*, 2006
ZAT12	AT5G59820	Transcription factor, Zn-finger TF	*Arabidopsis*		1	1	1				gain-of-function	Davletova *et al.*, 2005
			Arabidopsis								gain-of-function	Iida *et al.*, 2000
			Arabidopsis						1		gain-of-function	Rizhsky *et al.*, 2004
			Arabidopsis					1			gain-of-function	Vogel *et al.*, 2005
ZAT7	AT3G46090	Transcription factor, Zn-finger TF	*Arabidopsis*						1		gain-of-function	Rizhsky *et al.*, 2004
HRD/HARDY	AT2G36450	Transcription Factor, AP2/ERF-like	Rice	1	1						gain-of-function	Karaba *et al.*, 2007
			Arabidopsis	1	1						gain-of-function	Karaba *et al.*, 2007
NF-YB1	AT2G38880	Transcription Factor, Plant nuclear factor Y	*Arabidopsis*	1							gain-of-function	Nelson *et al.*, 2007
DREB2C	??	Transcription Factor (Drought-Responsive Element Binding protein)	*Arabidopsis*				1				gain-of-function	Lim *et al.*, 2007
Transport												
ALS3	unmapped	Aluminum sensitive 3, ABC transporter	*Arabidopsis*							1	gain-of-function	Gabrielson *et al.* 2006
ATM3	AT5G58270	ABC transporter	*Arabidopsis*							1	gain-of-function	Kim *et al.* 2006
BCB	AT5G20230	Blue copper-binding protein	*Arabidopsis*							1	gain-of-function	Ezaki *et al.* 2001
			Arabidopsis						1	1	gain-of-function	Ezaki *et al.* 2000
BOR1	AT2G47160	Boron transporter	*Arabidopsis*							1	gain-of-function	Kyoko *et al.* 2006
GLB1	AT2G16060	Non-symbiotic hemoglobin	*Arabidopsis*						1		gain-of-function	Yang *et al.*, 2005
MGT1	??	Mg++ transporter protein, membrane	*Nicotiana benthamiana*								gain-of-function	Deng *et al.*, 2006
MRP5	AT1G04120	ABC transporter	*Arabidopsis*	1							loss-of-function	Klein *et al.*, 2003
MTP11	AT2G39450	Golgi-localized manganese transporter that is involved in Mn tolerance	*Arabidopsis*							1	gain-of-function	Peiter *et al.* 2007
MTP3	AT3G58810	Zinc transporter (ZAT) family. Contributes to basic cellular Zn tolerance	*Arabidopsis*							1	gain-of-function	Arrivault *et al.* 2006
NHX1	AT5G27150	Vacuolar Na+/H+ antiporter	Cotton		1						gain-of-function	He *et al.*, 2005
			Wheat		1						gain-of-function	Xue *et al.*, 2004
			Arabidopsis		1						gain-of-function	Apse *et al.*, 1999
			Yeast		1						gain-of-function	Yokoi *et al.*, 2002
			Tall fescue		1						gain-of-function	Zhao *et al.* 2007
			Rapeseed		1						gain-of-function	Zhang *et al.*, 2001
NHX2	AT3G05030	Vacuolar Na+/H+ antiporter	Yeast		1						gain-of-function	Yokoi *et al.*, 2002
NHX5	AT1G54370	Vacuolar Na+/H+ antiporter	Yeast		1						gain-of-function	Yokoi *et al.*, 2002
PDR12	AT1G15520	ABC transporter	*Arabidopsis*							1	gain-of-function	Lee *et al.*, 2005
PDR8	AT1G59870	ABC transporter	*Arabidopsis*							1	gain-of-function	Kim *et al.*, 2007
PIP1;4	AT4G00430	Plasma membrane aquaporin	Tobacco	-1				1			gain-of-function	Jang *et al.*, 2007
			Arabidopsis	-1				1			gain-of-function	Jang *et al.*, 2007
PIP2;5	AT3G54820	Plasma membrane aquaporin	Tobacco	-1				1			gain-of-function	Jang *et al.*, 2007
			Arabidopsis	-1				1			gain-of-function	Jang *et al.*, 2007
SOS1	AT2G01980	Na+-H+ antiporter, membrane	*Arabidopsis*		1						gain-of-function	Shi *et al.*, 2003
SULTR1;2	AT1G78000	Sulfate transporter	*Arabidopsis*							1	loss-of-function	El Kassis *et al.*, 2007
VP/AVP1	AT1G15690	Vacuolar Na+/H+ antiporter / H+-PPases	*Arabidopsis*	1	1						gain-of-function	Gaxiola *et al.*, 2001
ZIF1	AT5G13740	Zn sequestration	*Arabidopsis*							1	loss-of-function	Haydon and Cobbett, 2007
CAX2	AT3G13320	Vacuolar Ca2+/H+ antiporter	Tobacco							1	gain-of-function	Korenkov *et al.*, 2007
CAX4	AT5G01490	Vacuolar Ca2+/H+ antiporter	Tobacco							1	gain-of-function	Korenkov *et al.*, 2007

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Other functions												
SBP1	AT1G45976	Selenium binding protein 1, S-ribonuclease binding protein SBP1	*Arabidopsis*							1	gain-of-function	Agalou *et al.*, 2005
ALX8	unmapped	Altered expression of APX2	*Arabidopsis*	1							gain of function	Rossel et al, 2006
ESK1	AT3G55990	Unknown protein	*Arabidopsis*					1			loss-of-function	Xin *et al.*, 2007
GPP2	AT5G57440	Haloacid dehalogenase-like hydrolase protein	*Arabidopsis*		1	1			1		gain-of-function	Caparrós-Martin *et al.*, 2007
ORE1	unmapped		*Arabidopsis*						1		loss-of-function	Woo *et al.*, 2004
PDC1,2	unmapped	Pyruvate decarboxylase	*Arabidopsis*								gain-of-function	Ismond *et al.* 2003
PST1	unmapped	Unmapped EMS mutant	*Arabidopsis*		1				1	1	loss-of-function	Tsugane *et al.*, 1999
TRG1	unmapped		*Arabidopsis*				1				loss-of-function	Tamura *et al.*, 2006
TRG2	unmapped		*Arabidopsis*				1				loss-of-function	Tamura *et al.*, 2006
CGS	AT3G01120	Cystathionine gamma-synthase, first committed step in methionine biosynthesis	Rapeseed							1	gain-of-function	Van Huysen *et al.*, 2003
SPS		Sucrose phosphate synthase	*Arabidopsis*					1			gain-of-function	Strand *et al.*, 2003
CHYB	AT4G25700	Beta-carotene hydroxylase	*Arabidopsis*								gain-of-function	Davison *et al.*, 2002
CESA8/IRX1/LEW2	AT4G18780	Cellulose synthase	*Arabidopsis*	1	1	1					loss-of-function	Chen *et al.* 2005
HAL3A	AT3G18030	Flavin mononucleotide flavoprotein (phosphopantothenoylcysteine decarboxylase activity)	*Arabidopsis*		1	1					gain-of-function	Espinosa-Ruiz *et al.* 1999
TMAC2	AT3G02140	Two or more ABREs-containing gene 2	*Arabidopsis*		1						gain-of-function	Huang and Wu, 2007
PCR1	AT1G54560	Myosin like protein	*Arabidopsis*							1	gain-of-function	Song *et al.*, 2004
PHYA (ars4ars5)	AT1G09570	Cytoplasmic red/far-red light photoreceptor	*Arabidopsis*							1	loss-of-function	Sung *et al.*, 2007
ADR1	AT1G33560	Encodes a NBS-LRR disease resistance protein that possesses N-terminal kinase subdomains	*Arabidopsis*	1	-1		-1				gain-of-function	Chini *et al.*, 2004
RCD1	AT1G32230	Radical-induced cell death 1	*Arabidopsis*						1		loss-of-function	Fujibe *et al.*, 2006

D, drought; S, salt; O, osmotic stress; H, heat stress; C/F, cold / freezing stress; Ox, oxidative stress; M, metal stress; 1 indicates tolerance; -1 indicates sensitivity

Supplementary Table S2. Stress-related expression clusters of the *Arabidopsis* stress tolerance genes

Locus	Description	Functional Class	Tolerance
CLUSTER A			
AT5G59820	RHL41 (RESPONSIVE TO HIGH LIGHT 41) transcription factor	Transcription	Salt, osmotic, freezing, heat, oxidative and light
AT4G17615	CBL1 (CALCINEURIN B-LIKE PROTEIN 1); calcium ion binding	Signaling	Drought, salt and freezing
AT1G27730	STZ (SALT TOLERANCE ZINC FINGER) transcription factor	Transcription	Drought, salt, osmotic and heat
AT5G20230	ATBCB (*ARABIDOPSIS* BLUE-COPPER-BINDING PROTEIN); copper ion binding	Transport	Oxidative and Metal (Al)
AT3G15500	ATNAC3 (*ARABIDOPSIS* NAC DOMAIN CONTAINING PROTEIN 55); transcription factor	Transcription	Drought
AT2G47190	MYB2 (myb domain protein 2) transcription factor	Transcription	Osmotic stress
AT3G22370	AOX1A (alternative oxidase 1A); alternative oxidase	Detoxification and redox control	Cold
AT1G15520	ATPDR12/PDR12 (PLEIOTROPIC DRUG RESISTANCE 12)	Transport	Metal (lead)
AT1G32640	ATMYC2 (JASMONATE INSENSITIVE 1); DNA binding / transcription factor	Transcription	Osmotic stress
AT1G52890	ANAC019 (*Arabidopsis* NAC domain containing protein 19); transcription factor	Transcription	Drought
AT3G14440	NCED3 (NINE-CIS-EPOXYCAROTENOID DIOXYGENASE3)	Hormone biosynthesis	Drought, salt and osmotic
no cluster assigned			
AT1G56600	ATGOLS2 (*ARABIDOPSIS THALIANA* GALACTINOL SYNTHASE 2)	Molecular protection	Drought
CLUSTER B			
AT3G19290	ABF4 (ABRE BINDING FACTOR 4) transcription factor	Transcription	Drought, salt
AT4G34000	ABF3/DPBF5 (ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING FACTOR 3) transcription factor	Transcription	Drought, salt
AT3G11410	AHG3/ATPP2CA (*ARABIDOPSIS THALIANA* PROTEIN PHOSPHATASE 2CA)	Protein metabolism/ stability	Cold
AT1G72770	HAB1 Involved in abscisic acid (ABA) signal transduction	Signaling	Drought
AT4G26080	ABI1 Involved in abscisic acid (ABA) signal transduction		
AT1G73500	ATMKK9 (*Arabidopsis thaliana* MAP kinase kinase 9); kinase	Signalling	Salt, osmotic
AT1G54100	Aldehyde dehydrogenase	Detoxification and redox control	Drought, salt
AT4G35790	ATPLDDELTA (*Arabidopsis thaliana* phospholipase D delta); phospholipase D	Lipid metabolism	Freezing
AT4G27410	RD26 (RESPONSIVE TO DESSICATION 26); transcription factor	Transcription	Drought
AT4G02380	SAG21 (SENESCENCE-ASSOCIATED GENE 21); LEA5	Molecular protection	Drought and oxidative
CLUSTER C			
AT5G45340	CYP707A3 (cytochrome P450, family 707, subfamily A, polypeptide 3); oxygen binding	Hormone biosynthesis	Drought
AT3G50970	LTI30, Dehydrin	Molecular protection	Cold/freezing
AT1G20440	COR47, Dehydrin	Molecular protection	Cold/freezing
CLUSTER D			
AT4G26850	VTC2 (VITAMIN C DEFECTIVE 2)	Metabolism	Heat + HL
AT1G75100	JAC1 (J-DOMAIN PROTEIN REQUIRED FOR CHLOROPLAST ACCUMULATION RESPONSE 1); heat shock protein binding	Molecular protection	Metal (Al)
AT5G16820	HSF3 (HEAT SHOCK FACTOR 3); DNA binding / transcription factor	Transcription	Heat
AT5G57440	GS1 (GLYCEROL-3-PHOSPHATASE 2); hydrolase	Metabolism	Salt, osmotic and oxidative
AT1G78290	Serine/threonine protein kinase, putative	Signaling	Drought and osmotic
AT1G15360	SHN1/WIN1 (SHINE1); DNA binding / transcription factor	Transcription	Drought
AT3G44110	ATJ3 (*Arabidopsis thaliana* DnaJ homologue 3)	Molecular protection	Heat
AT5G03740	HD2C (HISTONE DEACETYLASE 2C); nucleic acid binding / zinc ion binding	Transcription	Salt and drought
AT5G22060	ATJ2 (*Arabidopsis thaliana* DnaJ homologue 2)	Molecular protection	Heat
AT4G25130	Peptide methionine sulfoxide reductase, putative	Post-translational control	Oxidative and HL
no cluster assigned			
AT1G01720	ATAF1 (*Arabidopsis* NAC domain containing protein 2); transcription factor	Transcription	Drought
AT5G05410	DREB2A (DRE-BINDING PROTEIN 2A) transcription factor	Transcription	Drought, freezing and heat
AT3G02140	TMAC2 (TWO OR MORE ABRES-CONTAINING GENE 2)	Hormone response	Salt
CLUSTER E			
AT4G25480	DREB1A (DEHYDRATION RESPONSE ELEMENT B1A) transcription factor	Transcription	Drought, freezing, cold and salt
AT4G25470	CBF2 (FREEZING TOLERANCE QTL 4) transcription factor	Transcription	Drought, freezing and salt
AT4G25490	CBF1 (C-REPEAT/DRE BINDING FACTOR 1) transcription factor	Transcription	Drought, freezing, cold and salt
AT5G51990	CBF4/DREB1D (C- REPEAT-BINDING FACTOR 4) transcription factor	Transcription	Drought, freezing
AT2G42540	COR15A (COLD-REGULATED 15A)	Response to stress	Freezing
CLUSTER F			
AT5G66400	RAB18, dehydrin	Molecular protection	Cold/freezing
AT3G24650	ABI3 Transcription factor	Transcription	Cold/freezing
AT3G56350	Mn superoxide dismutase	Detoxification and redox control	Salt
AT3G30775	ERD5, proline dehydrogenase	Molecular protection	Salt, cold/freezing
no cluster assigned			
AT4G02390	Poly(ADP-ribose) polymerase 2	DNA repair/replication	Drought, heat, oxidative
CLUSTER G			
AT1G74310	ATHSP101 (HEAT SHOCK PROTEIN 101); ATP binding / ATPase	Molecular protection	Heat
AT2G26150	ATHSFA2 (*Arabidopsis thaliana* heat shock transcription factor A2) transcription factor	Transcription	Heat, salt, osmotic, oxidative stress and Combined HL+HS+MV
AT3G24500	ATMBF1C/MBF1C (MULTIPROTEIN BRIDGING FACTOR 1C) transcription factor	Transcription	Heat, salt, osmotic stress, HL, pathogen
AT5G12030	AT-HSP17.6A (*Arabidopsis thaliana* heat shock protein 17.6A)	Molecular protection	Salt, drought

Supplementary Table S3. Stress-tolerant transgenic crop species

Gene	Molecular Function	Source	Species	D	S	O	H	C/F	Ox	M	Construct	Reference
Detoxification and Redox control												
MT2a	Metallothionein	*Arabidopsis*	Bean							1	gain-of-function	Lee *et al.*, 2004
MT3	Metallothionein	*Arabidopsis*	Bean							1	gain-of-function	Lee *et al.*, 2004
APX	Ascorbate peroxidase	Pea	Cotton					1			gain-of-function	Kornyeyev *et al.* 2003
		Pea	Tomato		1			1			gain-of-function	Wang *et al.*, 2005
CAT	Catalase	Wheat	Rice					1			gain-of-function	Matsumura *et al.*, 2002
GST	Glutathione S-transferase	*Suaeda salsa*	Rice		1			1	1		gain-of-function	Zhao and Zhang, 2006
SOD	Mn superoxide dismutase	Pea	Rice	1							gain-of-function	Wang *et al.*, 2005
		Wheat	Rapeseed						1	1	gain-of-function	Basu *et al.*, 2001
Hormone Biosynthesis												
ACC	ACC deaminase	bacterial	Rapeseed							1	gain-of-function	Stearns *et al.*, 2005
ACS6	1-aminocyclopropane-1-carboxylate (ACC) synthase	Maize	Maize	1							loss-of-function	Young *et al.*, 2004
Lipid biosynthesis/metabolism/signaling												
FAD	Fatty acid desaturase	Rice	Rice				1				loss-of-function	Sohn and Back, 2007
GPAT	Glycerol-3-phosphate acyltransferase of chloroplasts	*Arabidopsis*	Rice					1			gain-of-function	Ariizumi *et al.*, 2002
		Tomato	Tomato					1			gain-of-function	Sui *et al.*, 2007
		Spinach	Rice					1			gain-of-function	Ariizumi *et al.*, 2002
Molecular Protection												
HVA1	Group 3 LEA protein gene	Barley	Rice	1							gain-of-function	Babu *et al.*, 2004
		Barley	Oat		1	1					gain-of-function	Maqbool *et al.*, 2002
		Barley	Oat		1						gain-of-function	Oraby *et al.*, 2005
		Barley	Rice	1	1						gain-of-function	Rohila *et al.* 2002
		Barley	Wheat	1							gain-of-function	Sivamani *et al.* 2000
		Barley	Wheat	1							gain-of-function	Bahieldin *et al.*, 2005
LEA3	Lea protein	Rice	Rice	1							gain-of-function	Xiao *et al.*, 2007
TPS+TPP fusion		bacterial	Rice	1	1			1			gain-of-function	Jang *et al.*, 2003
TPS1	Trehalose-6-phosphate synthase	Yeast	Tomato	1	1				1		gain-of-function	Cortina and Culiáñez-Macià, 2005
OtsA + OtsB fusion	Trehalose-6-phosphate synthase (trehalose synthesis)	bacterial	Rice	1	1			1			gain-of-function	Garg *et al.*, 2002
BetA	Choline dehydrogenase (glycinebetaine synthesis)	bacterial	Maize	1							gain-of-function	Ruidang *et al.*, 2004
CMO	Choline monooxygenase (glycine betaine synthesis)	Spinach	Rice		1						gain-of-function	Shirasawa *et al.*, 2006
CodA /COX	Choline oxidase (glycine betaine synthesis)	bacterial	Tomato					1	1		gain-of-function	Park *et al.*, 2007
		bacterial	Rice		1						gain-of-function	Mohanty *et al.*, 2003
		bacterial	Rice		1			1			gain-of-function	Sakamoto *et al.* 1998
		bacterial	Tomato					1	1		gain-of-function	Eung-Jun *et al.*, 2004
COIN	Cold Inducible Zinc finger protein involved in inducing proline levels	Rice	Rice	1	1			1			gain-of-function	Liu *et al.*, 2007
MtlD	Mannitol-1-phosphate dehydrogenase (mannitol synthesis)	bacterial	Wheat	1	1						gain-of-function	Abebe *et al.*, 2003
OAT	Ornithine-delta-aminotransferase	*Arabidopsis*	Rice	1	1						gain-of-function	Wu *et al.*, 2005
P5CR	Pyrroline carboxylate reductase (proline accumulation)	*Arabidopsis*	Soybean	1			1				gain-of-function	Kocsy *et al.*, 2005
		Arabidopsis	Soybean		1		1				gain-of-function	De Ronde *et al.* 2001
		Arabidopsis	Soybean	1							gain-of-function	De Ronde *et al.*, 2004
P5CS	Pyrroline carboxylate synthase(proline synthesis)	*Arabidopsis*	Potato		1						gain-of-function	Hmida-Sayari *et al.*, 2005
		?	Rice	1	1						gain-of-function	Zhu *et al.* 1998
		?	Rice	1	1						gain-of-function	Su and Wu, 2004
		Vigna aconitifolia	Rice		1						gain-of-function	Hong Zong Lie *et al.*, 2000
		Vigna aconitifolia	Wheat	1							gain-of-function	Vendruscolo *et al.*, 2007
			Sugarcane	1							gain-of-function	Molinari *et al.*, 2007
HSP101	Heat shock protein	*Arabidopsis*	Rice				1				gain-of-function	Katiyar-Agarwal *et al.* 2003

Gene	Molecular Function	Source	Species	D	S	O	H	C/F	Ox	M	Construct	Reference
Signaling												
CIPK03	Calcineurin B-like protein-interacting protein kinases	Rice	Rice					1			gain-of-function	Xiang *et al.*, 2007
CIPK12	Calcineurin B-like protein-interacting protein kinases	Rice	Rice	1							gain-of-function	Xiang *et al.*, 2007
CIPK15	Calcineurin B-like protein-interacting protein kinases	Rice	Rice		1						gain-of-function	Xiang *et al.*, 2007
NPK1	Mitogen-activated protein kinase	Tobacco	Maize					1			gain-of-function	Shou *et al.*, 2004
		Tocacco	Maize	1							gain-of-function	Shou *et al.*, 2004
CALCINEURIN	Ca2+- and calmodulin-dependent serine/threonine phosphatase	Mice	Rice		1						gain-of-function	Ma *et al.*, 2005
CDPK7	Ca2+-dependent protein kinase	Rice	Rice	1	1			1			gain-of-function	Saijo *et al.* 2000
NDPK2	NDP kinases	*Arabidopsis*	Potato				1		1		gain-of-function	Tang *et al.*, 2007
GSK3/SHAGGY/BIN2/SK21	Glycogen synthase kinase	Rice	Rice	1	1		1	1			loss-of-function	Koh *et al.*, 2007
Transcription												
ABF3	Transcription Factor (binds ABA responsive elements)	*Arabidopsis*	Rice	1							gain-of-function	Oh *et al.*, 2005
CBF1 / DREB1B	Transcription Factor (Cold binding factor, Drought-Responsive Element Binding protein)	*Arabidopsis*	Potato					1			gain-of-function	Pino *et al.*, 2007
		Arabidopsis	Tomato					1			gain-of-function	Hsieh *et al.* 2002
		Rice	Rice	1	1			1			gain-of-function	Ito *et al.* 2006
CBF15	Transcription Factor (Cold binding factor, Drought-Responsive Element Binding protein)	Rapeseed	Rapeseed					1			gain-of-function	Savitch *et al.*, 2005
CBF17	Transcription Factor (Cold binding factor, Drought-Responsive Element Binding protein)	Rapeseed	Rapeseed					1			gain-of-function	Savitch *et al.*, 2005
CBF3 / DREB1A	Transcription Factor (Cold binding factor, Drought-Responsive Element Binding protein)	*Arabidopsis*	Potato					1			gain-of-function	Pino *et al.*, 2007
		Arabidopsis	Rice	1	1			1			gain-of-function	Oh *et al.*, 2005
		Arabidopsis	Wheat	1							gain-of-function	Pellegrineschi *et al.*, 2004
		Rice	Rice	1	1			1			gain-of-function	Ito *et al.* 2006
CBF4/DREB1D	Transcription Factor (Cold binding factor, Drought-Responsive Element Binding protein)	Barley	Rice	1	1			1			gain-of-function	Oh *et al.*, 2007
EREBP1	Transcription factor AP2/EREBP	Potato	Potato		1			1			gain-of-function	Lee *et al.*, 2007
HRD/HARDY	Transcription Factor, AP2/ERF-like	*Arabidopsis*	Rice	1	1						gain-of-function	Karaba *et al.*, 2007
MYB4	Transcription factor	Rice	Tomato	1							gain-of-function	Vannini *et al.*, 2007
NAC6	Transcription Factor, NAC domain	Rice	Rice	1	1						gain-of-function	Nakashima *et al.*, 2007
NF-YB2	Transcription Factor, Plant nuclear factor Y	Maize	Maize	1							gain-of-function	Nelson *et al.*, 2007
PIF1	Transcription Factor Cys-2/His-2 zinc finger	Pepper	Tomato					1			gain-of-function	Seong *et al.*, 2007
SNAC1	Transcription factor STRESS-RESPONSIVE NAC 1	Rice	Rice	1	1						gain-of-function	Hu *et al.*, 2006
Transport												
ALMT1	Aluminum-activated malate transporter	Wheat	Barley							1	gain-of-function	Delhaize *et al.*, 2004
		Wheat	Rice							1	gain-of-function	Takayuki *et al.*, 2004
		Wheat	Wheat							1	gain-of-function	Takayuki *et al.*, 2004
HAL1	Na/K transporter	Yeast	Tomato		1						gain-of-function	Rus *et al.*, 2001
		Yeast	Watermelon		1						gain-of-function	Ellul *et al.* 2003
HAL2	Na/K transporter	Yeast	Tomato		1						gain-of-function	Arrillaga *et al.* 1998
HKT1	Potassium transporter	Wheat	Wheat		1						loss-of-function	Laurie *et al.*, 2002
KAT1	Shaker family K+ channel	Rice	Rice		1						gain-of-function	Obata *et al.*, 2007
NHA-A	Na^+/H^+ antiporter	bacterial	Rice	1	1						gain-of-function	Wu *et al.*, 2005
NHX1	Vacuolar Na+/H+ antiporter	*Arabidopsis*	Cotton		1						gain-of-function	He *et al.*, 2005
		Arabidopsis	Wheat		1						gain-of-function	Xue *et al.*, 2004
		Rice	Grass		1						gain-of-function	Wu *et al.*, 2005
		Rice	Rice		1						gain-of-function	Fukuda *et al.*, 2004
		Suaeda salsa	Rice		1						gain-of-function	Zhao *et al.* 2006
		Arabidopsis	Rapeseed		1						gain-of-function	Zhang *et al.*, 2001
RWC3	Aquaporin	Rice	Rice			1					gain-of-function	Lian *et al.*, 2004
SOS1/SOD2	Vacuolar Na+/H+ antiporter / H+-PPases	Yeast	Rice		1						gain-of-function	Zhao *et al.* 2006

Gene	Molecular Function	Source	Species	D	S	O	H	C/F	Ox	M	Construct	Reference
Other Functions												
GDH	Glutamate Dehydrogenase	bacterial	Rice	1							gain-of-function	Lightfoot *et al.*, 2007
GS2	Chloroplastic glutamine synthetase	Rice	Rice		1			1			gain-of-function	Hoshida *et al.* 2000
		Rice	Rice		-1			-1			loss-of-function	Hoshida *et al.* 2000
PPO	Polyphenol oxidase	Potato	Tomato	1							loss-of-function	Thipyapong *et al.*, 2004
SAMDC	S-adenosylmethioninedecarboxylase (polyamine synthesis)	Tritordeum	Rice		1						gain-of-function	Malabika and Wu, 2002
ADC	Arginine decarboxylase in involved in putrescine biosynthesis	Datura stramonium	Rice	1							gain-of-function	Capell *et al.*, 2004
		Oat	Rice		1						gain-of-function	Roy and Wu, 2001
		Oat	Rice	1							gain-of-function	Capell *et al.* 1998
BCL-xL	antiapoptotic	Human	Tomato					1			gain-of-function	Xu *et al.*, 2004
CED9	antiapoptotic	Nematode	Tomato					1			gain-of-function	Xu *et al.*, 2004
RF1	Fertility restorer	Rice	Rice					1			gain-of-function?	Toshiyuki and Hidemasa, 2005
OSMOTIN	Osmotin protein accumulation	Tobacco	Olive trees					1			gain-of-function	Angeli and Altamura, 2007
PARP1	Poly(ADP-ribose) polymerase	*Arabidopsis*	Rapeseed	1			1		1		loss-of-function	Block *et al.*, 2005
PARP2	Poly(ADP-ribose) polymerase	*Arabidopsis*	Rapeseed	1			1		1		loss-of-function	Block *et al.*, 2005
CIT1	Mitochondrial citrate synthase, condensation reaction of the two-carbon acetate residue from acetyl coenzyme A and a molecule of four-carbon oxaloacetate to form the six-carbon citrate.	Yeast	Rapeseed							1	gain-of-function	Anoop *et al.* 2003
SBP	Sedoheptulose-1,7-bisphosphatase	Rice	Rice		1						gain-of-function	Feng *et al.*, 2007
FTB/ERA1	Farnesyltransferase	*Arabidopsis*	Rapeseed	1							loss-of-function	Wang *et al.*, 2005
OCPI1	Chymotrypsin inhibitor-like 1	Rice	Rice	1							gain-of-function	Huang *et al.*, 2007
SacB	Levansucrase, a fructosyltransferase	bacterial	Sugar beet	1							gain-of-function	Pilon-smits *et al.* 1999

D, drought; S, salt; O, osmotic stress; H, heat stress; C/F, cold / freezing stress; Ox, oxidative stress; M, metal stress; 1 indicates tolerance; -1 indicates sensitivity

Supplementary Table S4. Selected cDNA-AFLP fragments

VIGS nr	Description	cDNA-AFLP tag	*E. coli* pDONR207	pTV00::GW2 for VIGS in *N. benthamiana*	TRVRNA2 for VIGS in tomato
H_2O_2-induced genes					
1	unknown, embryo-abundant protein EMB	BC11-M4-009	yes	yes	
2	No significant match	BC1-M23-016	yes	yes	
3	putative protein, stellacyanin	BC1-M44-048	yes	yes	
4	plastidic ATP/ADP-transporter	BC2-M14-039	yes	yes	
5	No significant match	BC2-M31-045	yes	yes	
6	No significant match	BC2-M44-067	yes	yes	
7	scarecrow gene regulator, putative	BC3-M14-016	yes	yes	
8	No significant match	BC3-M32-060	yes	yes	
9	shock protein SRC2 homolog; unknown protein	BC3-M41-035	yes	yes	
10	DNA-binding protein 4, contains WRKY domain	BC4-M21-034	yes	no	
11	low molecular weight HSP precursor (clone Hsp22.3)	BC4-M42-015	yes	yes	
12	BYPASS	BC4-M44-046	yes	yes	yes
13	WIZZ	BT4-M32-061	yes	yes	
14	No significant match	BC1-M23-048	yes	yes	
15	No significant match	BC2-M12-032	yes	yes	
16	photosystem I antenna protein	BC2-M21-025	yes	yes	
17	unknown	BC2-M32-008	yes	yes	
18	unknown	BC31-M2-034	yes	yes	
19	putative protein + putative chloroplast nucleoid DNA binding protease	BC3-M22-004	yes	yes	
20	unknown	BC3-M33-028	yes	yes	
21	No significant match	BC43-M1-028	yes	yes	
22	MRP protein, putative	BC4-M22-043	yes	yes	
23	unknown	BC4-M42-022	yes	yes	
24	No significant match	BT11-M4-032	yes	yes	
25	No significant match	BC11-M4-054	no	no	
26	No significant match	BC1-M24-036	yes	yes	
27	No significant match	BC2-M13-004	yes	yes	
28	cystathionine gamma-synthase isoform 2 (CgS2)	BC2-M22-011	yes	yes	
29	xylosidase, glycosyl hydrolase family 3	BC2-M32-017	yes	yes	
30	No significant match	BC33-M2-042	yes	yes	
31	No significant match	BC3-M24-036	yes	yes	
32	putative protein + AP2 (sequenced twice)	BC3-M33-063	yes	yes	
33	No significant match	BC43-M1-063	yes	yes	
34	No significant match	BC4-M32-009	yes	yes	
35	cytochrome P450	BC4-M42-026	yes	yes	
36	No significant match	BT1-M11-055	yes	yes	
37	ACC-oxidase	BC11-M4-061	yes	yes	
38	rubisco, chain S	BC1-M32-023	yes	yes	
39	unknown	BC2-M13-011	yes	yes	
40	D1 CtpA arboxy-terminal protease, putative	BC2-M22-012	yes	yes	
41	ADP-glucose pyrophosphorylase large subunit	BC2-M42-011	yes	yes	
42	cathepsin B cysteine proteinase, putative	BC34-M2-004	yes	yes	
43	No significant match	BC3-M24-037	yes	no	
44	AP2 domain containing protein, putative	BC3-M33-088	yes	yes	
45	No significant match	BC43-M1-095	yes	yes	
46	HSP70	BC4-M32-017	yes	yes	
47	unknown	BC4-M42-042	yes	yes	yes
48	No significant match	BT1-M13-069	yes	yes	
49	sulfate adenylyltransferase	BC1-M12-008	yes	yes	
50	ubiquitin-conjugating enzyme, putative	BC1-M41-018	yes	yes	yes
51	TnpB [Bacteriophage phiE125]	BC2-M13-013	yes	yes	
52	No significant match	BC2-M22-020	yes	yes	yes
53	chitinase, class V	BC2-M42-015	yes	yes	
54	putative protein, fasciclin-like arabinogalactan-protein	BC34-M2-023	yes	yes	
55	threonyl-tRNA synthetase, mitochondrial precursor	BC3-M24-052	yes	yes	yes
56	unknown	BC3-M33-106	yes	yes	yes
57	ethylene-responsive protein 2	BC4-M12-025	yes	yes	
58	unknown	BC4-M33-051	yes	yes	
59	unknown	BC4-M43-005	yes	yes	
60	unknown	BT1-M21-020	yes	yes	yes
61	S-adenosyl-methionine-sterol-C-methyltransferase homolog	BC1-M12-071	yes	yes	
62	FtsH protease, putative	BC1-M43-002	yes	yes	
63	No significant match	BC2-M13-038	yes	yes	yes
64	cystathionine gamma-synthase isoform 1	BC2-M22-028	yes	yes	yes
65	In2-1 protein	BC2-M42-018	yes	yes	yes
66	CCR4-associated factor	BC34-M2-035	yes	yes	yes
67	unknown	BC3-M31-012	yes	no	
68	pyridine nucleotide-disulphide oxidoreductase class-I	BC3-M34-001	yes	no	
69	UDP-Glucose:protein transglucosylase	BC4-M12-032	yes	no	
70	Lil3 protein (*Arabidopsis*)	BC4-M34-002	yes	no	
71	unknown	BC4-M43-028	yes	no	

Supplementary Table S4

VIGS nr	Description	cDNA-AFLP tag	*E. coli* pDONR207	pTV00::GW2 for VIGS in *N. benthamiana*	TRVRNA2 for VIGS in tomato
72	protein kinase, putative	BT1-M21-024	yes	yes	yes
73	cytosolic class I small HSP17.5	BC1-M22-006	yes	no	
74	AP2 domain containing protein	BC1-M43-024	yes	yes	yes
75	chlorophyll A-B binding protein 91R, chloroplast precursor	BC2-M14-017	yes	yes	
76	polyubiquitin UBQ10, putative	BC2-M23-040	yes	yes	
77	No significant match	BC2-M42-022	yes	yes	yes
78	scarecrow gene regulator, putative	BC3-M13-015	yes	yes	
79	No significant match	BC3-M32-022	yes	yes	yes
80	unknown	BC3-M34-009	yes	no	
81	No significant match	BC4-M14-069	yes	yes	yes
82	esterase, putative	BC4-M34-045	yes	yes	yes
83	glucan phosphorylase	BC4-M43-030	yes	yes	
84	No significant match	BT1-M21-044	yes	yes	
85	No significant match	BC1-M22-037	yes	no	
86	No significant match	BC1-M43-038	yes	yes	
87	narf-like protein	BC2-M14-026	yes	yes	yes
88	No significant match	BC2-M31-017	yes	yes	
89	No significant match	BC2-M43-030	yes	no	
90	calmodulin-binding HSP	BC3-M13-022	yes	yes	yes
91	No significant match	BC3-M32-029	yes	yes	
92	symbiosis-related protein	BC3-M41-023	yes	yes	
93	unknown	BC4-M21-016	yes	yes	yes
94	unknown	BC4-M41-009	yes	yes	
95	cytokinin inducible gene	BC4-M44-036	yes	yes	
96	26S proteasome regulatory particle non-ATPase subunit2a	BT1-M21-048	yes	yes	yes
97	cytochrome b6 apoprotein	BT1-M22-007	yes	yes	yes
98	unknown	BT1-M33-050	yes	yes	
99	cytochrome C	BT42-M1-010	yes	yes	
100	unknown	BT2-M14-018	yes	yes	
101	chlorophyll A-B binding protein 40, chloroplast precursor	BT2-M23-004	yes	yes	yes
102	WRKY1	BT4-M32-068	yes	yes	
103	small HSP class CIII	BT2-M41-008	yes	yes	yes
104	No significant match	BT31-M2-063	yes	yes	
105	Low molecular weight HSP	BT3-M22-004	yes	yes	yes
106	No significant match	BT4-M11-006	yes	yes	
107	polyubiquitin	BT4-M23-022	yes	yes	
108	unknown	BT4-M33-006	yes	yes	yes
109	putative protein 3- potato transposon Tst1	BT1-M31-024	yes	yes	
110	senescence-associated protein 12, putative	BT1-M34-032	yes	yes	
111	No significant match	BT2-M11-021	yes	yes	
112	unknown	BT2-M14-019	yes	no	
113	No significant match	BT2-M23-033	yes	yes	
114	RNA-binding protein RNP1 precursor (chloroplast)	BT2-M31-037	yes	yes	
115	unknown	BT2-M41-034	yes	yes	
116	probable 12-oxophytodienoate reductase	BT32-M3-011	yes	yes	
117	No significant match	BT3-M22-055	yes	yes	
118	unknown	BT4-M11-031	yes	yes	
119	No significant match	BT4-M23-026	yes	yes	yes
120	putative carrier protein	BT4-M33-007	yes	yes	
121	histone H2B	BT1-M31-028	yes	yes	
122	No significant match	BT1-M34-037	yes	yes	yes
123	eukaryotic cap-binding protein	BT2-M11-026	yes	yes	
124	unknown	BT2-M21-001	yes	yes	
125	unknown	BT2-M24-002	yes	yes	
126	unknown	BT2-M32-030	yes	yes	
127	CAB7 light harvesting chlorophyll a/b-binding protein	BT2-M42-024	yes	yes	
128	No significant match	BT34-M1-054	yes	yes	
129	putative oxalyl-CoA decarboxylase	BT3-M31-010	yes	yes	
130	HSP82	BT4-M21-010	yes	yes	
131	cytochrome P450-dependent fatty acid hydroxylase	BT4-M31-027	yes	yes	
132	3-oxoacyl-[acyl-carrier protein] reductase, chloroplast precursor	BT4-M33-010	yes	yes	
133	S-receptor kinase (SRK)	BT1-M31-038	yes	yes	
134	ABC transporter, putative	BT1-M43-037	yes	yes	
135	DnaJ-like protein	BT2-M12-010	yes	yes	
136	heat shock transcription factor HSF5	BT2-M21-052	yes	yes	
137	No significant match	BT2-M24-012	yes	yes	
138	myb factor	BT2-M33-006	yes	yes	
139	No significant match	BT2-M42-070	yes	yes	
140	No significant match	BT34-M2-062	yes	yes	
141	HSP100/ClpB, putative	BT3-M41-009	yes	yes	
142	ferredoxin--nitrite reductase	BT4-M21-017	yes	yes	
143	lycopene epsilon cyclase	BT4-M31-041	yes	yes	
144	prohibitin 1-like protein	BT4-M33-013	yes	yes	
145	unknown	BT1-M31-043	yes	yes	

VIGS nr	Description	cDNA-AFLP tag	*E. coli* pDONR207	pTV00::GW2 for VIGS in *N. benthamiana*	TRVRNA2 for VIGS in tomato
146	Avr9 elicitor response protein, putative	BT1-M43-043	yes	yes	
147	40S ribosomal protein S8	BT2-M12-050	yes	yes	
148	unknown	BT2-M22-005	yes	yes	
149	resistance complex protein I2C-1	BT2-M24-021	yes	yes	
150	unknown	BT2-M34-005	yes	yes	
151	retrotransposon Tnt1 dp51tr long terminal repeat	BT2-M43-007	yes	yes	
152	No significant match	BT34-M2-063	yes	yes	
153	methionine S-methyltransferase	BT21-M1-039	yes	yes	
154	No significant match	BT4-M21-029	yes	yes	
155	No significant match	BT4-M31-045	yes	yes	
156	No significant match	BT4-M33-027	yes	yes	
157	No significant match	BT1-M32-050	yes	no	
158	No significant match	BT1-M44-060	yes	yes	
159	No significant match	BT2-M12-051	yes	no	
160	glucosyltransferase NTGT3	BT2-M22-012	yes	yes	
161	unknown	BT2-M24-025	yes	yes	
162	No significant match	BT2-M34-037	yes	no	
163	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase (Vitamin-B12-independent methionine synthase isozyme)	BT2-M43-028	yes	no	
164	No significant match	BT34-M2-065	yes	yes	
165	pyruvate kinase, putative	BT42-M1-030	yes	yes	
166	phenylalanine ammonia-lyase	BT4-M21-037	yes	yes	
167	unknown	BT4-M32-050	yes	yes	
168	light harvesting chlorophyll a/b-binding protein	BT4-M33-031	yes	yes	
169	No significant match	BT1-M33-019	yes	no	
170	alanine aminotransferase, putative	BT21-M1-028	yes	yes	
171	ATP:citrate lyase	BT2-M13-003	yes	yes	
172	elongation factor-1 alpha	BT2-M22-023	yes	no	
173	putative protein, Pto kinase interactor	BT2-M24-028	yes	yes	
174	5-epi-aristolochene synthase	BT2-M41-004	yes	no	
175	receptor-like protein kinase, putative	BT31-M2-002	yes	yes	
176	unknown	BT34-M3-016	yes	yes	
177	unknown	BT42-M1-049	yes	yes	
178	lipase, putative	BT4-M23-015	yes	yes	
179	AAA-type ATPase-like protein	BT4-M32-056	yes	no	
180	glyceradehyde-3-phosphate dehydrogenase	BT4-M34-005	yes	no	
181	No significant match	BT1-M33-041	yes	yes	
182	ubiquitin-conjugating enzyme UBC7, putative	BT21-M1-035	yes	yes	
183	retroelement, putative	BT2-M13-043	yes	yes	
184	glutaredoxin	BT2-M22-030	yes	yes	
185	rubisco small subunit pseudogene	BT2-M24-050	yes	no	
186	unknown	BT2-M41-007	yes	no	
187	Tetrafunctional protein of glyoxysomal fatty acid beta-oxidation	BT31-M2-041	yes	yes	
188	pantothenate kinase, putative	BT3-M11-006	yes	yes	
189	No significant match	BT44-M2-004	yes	yes	
190	No significant match	BT4-M23-016	yes	yes	
191	No significant match	BT4-M32-067	yes	yes	
192	putative carboxyl-terminal peptidase	BT4-M34-033	yes	yes	
193	unknown	BT4-M43-021	yes	yes	
194	auxin-induced protein	BT4-M43-033	yes	yes	
195	dynamin protein ADL2, putative	BC11-M4-001	yes	yes	
196	putative protein + putative transposase	BC1-M22-032	yes	yes	
197	ELI3 (aromatic alcohol:NADP(+) oxidoreductase)	BC2-M13-001	yes	yes	
198	unknown	BC2-M14-024	yes	no	
199	unknown	BC2-M31-015	yes	yes	
200	unknown	BC2-M31-062	yes	no	
201	clathrin-coat assembly protein, putative	BC2-M32-005	yes	yes	
202	unknown	BC2-M32-015	yes	yes	
203	cytokinin up-regulated gene, fiber protein E6 protein kinase (cotton)	BC2-M34-032	yes	yes	
204	protein phosphatase 2C	BC2-M43-031	yes	yes	
205	unknown	BC3-M13-002	yes	no	
206	ethylene-responsive transcription factor ERF1	BC3-M14-076	yes	yes	
207	glucosyltransferase NTGT2	BC3-M21-013	yes	no	
208	receptor-like protein kinase, putative	BC3-M33-027	yes	no	
209	3-hydroxy-3-methylglutaryl-coenzyme A reductase	BC3-M34-021	yes	no	
210	unknown, elicitor-responsive gene 4	BC3-M34-052	yes	yes	
211	unknown	BC4-M14-020	yes	no	
212	subtilisin serine protease, putative	BC4-M22-045	yes	yes	
213	No significant match	BC4-M24-025	yes	yes	
214	No significant match	BC4-M41-059	yes	yes	
215	glycosylasparaginase, putative	BC4-M43-015	yes	yes	
216	No significant match	BT1-M11-047	yes	yes	
217	galactinol synthase, putative	BT1-M21-038	yes	yes	
218	No significant match	BT1-M23-046	yes	yes	

Supplementary Table S4

VIGS nr	Description	cDNA-AFLP tag	*E. coli* pDONR207	pTV00::GW2 for VIGS in *N. benthamiana*	TRVRNA2 for VIGS in tomato
219	No significant match	BT1-M24-006	yes	yes	
220	No significant match	BT1-M41-015	yes	yes	
221	glutathione S-transferase (Auxin-induced protein), putative	BT1-M43-004	yes	yes	
222	unknown	BT1-M43-024	yes	no	
223	unknown	BT2-M12-008	yes	no	
224	unknown	BT2-M23-011	yes	yes	
225	unknown	BT2-M31-030	yes	yes	
226	myb, typical P-type R2R3	BT34-M2-030	yes	yes	
227	No significant match	BT3-M23-039	yes	yes	
228	unknown	BT3-M41-004	no	no	
229	6-phosphogluconate dehydrogenase	BT4-M13-009	no	no	
230	ubiquitin RiP-20	BT4-M23-014	yes	yes	
231	unknown	BT4-M34-003	yes	yes	
232	BCS1 protein-like protein	BT4-M34-027	yes	yes	
233	unknown	BT4-M43-018	yes	yes	
234	nam-like protein 10	BT4-M44-030	yes	yes	
Abscisic acid and/or stress-responsive genes					
ABA1	NRK1	AB055515	yes	yes	yes
ABA2	NQK1	AB055514	yes	yes	yes
ABA3	Tsi1	AF058827	yes	yes	
ABA4	PK11-C1	U73938	yes	yes	
ABA5	Pin-I	K03290	yes	yes	
ABA6	Pin-II	K03291	yes	yes	
ABA7	LTP	D13952	yes	yes	
ABA8	PK11-C5	U73939	yes	yes	
ABA9	C12	AF258810	yes	yes	

Supplementary Table S5. SOS1-dependent genes

Locus	Description	Probeset	Fold Change	P value
UPREGULATED GENES				
AT5G23240; AT5G23235	[AT5G23240, DNAJ heat shock N-terminal domain-containing protein]	249850_at	25.66	2.07E-04
AT1G07050	CONSTANS-like protein-related	256060_at	21.86	1.28E-03
AT5G62360	invertase/pectin methylesterase inhibitor family protein	247478_at	15.08	4.99E-05
AT3G22231	PCC1 (PATHOGEN AND CIRCADIAN CONTROLLED 1)	256766_at	13.14	2.84E-05
AT5G24470	APRR5 (PSEUDO-RESPONSE REGULATOR 5); transcription regulator	249741_at	12.74	6.47E-06
AT2G21660	ATGRP7 (COLD, CIRCADIAN RHYTHM, AND RNA BINDING 2); RNA binding / double-stranded DNA binding / single-stranded DNA binding	263548_at	12.38	2.68E-03
AT3G43100	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT3G30550.1); similar to Nucleic acid-binding, OB-fold [Medicago truncatula] (GB:ABD32456.1); contains InterPro domain Nucleic acid-binding, OB-fold, subgroup; (InterPro:IPR012340); contains InterPro	252729_at	9.02	7.91E-19
AT5G60100	APRR3 (PSEUDO-RESPONSE REGULATOR 3); transcription regulator	247668_at	8.38	1.35E-03
AT5G36230	eIF4-gamma/eIF5/eIF2-epsilon domain-containing protein	246621_at	8.30	2.55E-16
AT2G39920	acid phosphatase class B family protein	267361_at	7.71	2.83E-07
AT1G56300	DNAJ heat shock N-terminal domain-containing protein	256221_at	7.00	3.96E-03
AT5G48250	zinc finger (B-box type) family protein	248744_at	6.84	1.76E-03
AT2G21130	peptidyl-prolyl cis-trans isomerase / cyclophilin (CYP2) / rotamase	264019_at	6.80	1.31E-04
AT4G16146	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT1G69510.2); similar to negatively light-regulated protein, putative, expressed [Oryza sativa (japonica cultivar-group)] (GB:ABA97694.2); contains InterPro domain Lg106-like; (InterPro:IPR012482)	245319_at	6.30	1.50E-04
AT2G42530	cold-responsive protein / cold-regulated protein (cor15b)	263495_at	6.16	2.38E-04
AT2G40080	ELF4 (EARLY FLOWERING 4)	267364_at	5.91	2.91E-03
AT4G04330	similar to unnamed protein product [Ostreococcus tauri] (GB:CAL56420.1); similar to Os08g0425200 [Oryza sativa (japonica cultivar-group)] (GB:NP_001061837.1)	255331_at	5.88	1.73E-03
AT3G22240	unknown protein	256617_at	5.54	4.41E-05
AT5G59570; AT3G46640	[AT5G59570, myb family transcription factor];[AT3G46640, PCL1 (PHYTOCLOCK 1); DNA binding / transcription factor]	252475_s_at	5.13	1.20E-02
AT1G11210	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT1G11220.1); similar to fiber expressed protein [Gossypium hirsutum] (GB:AAY85179.1); similar to cotton fiber expressed protein 1 [Gossypium hirsutum] (GB:AAC33276.1); contains InterPro domain Prote	262452_at	4.95	1.30E-05
AT2G22450	riboflavin biosynthesis protein, putative	264045_at	4.76	2.37E-03
AT3G07650	COL9 (CONSTANS-LIKE 9); transcription factor/ zinc ion binding	259244_at	4.60	4.95E-02
AT2G15890	MEE14 (maternal effect embryo arrest 14)	265478_at	4.52	3.18E-02
AT3G05800	transcription factor	258742_at	4.47	4.41E-04
AT1G51090	heavy-metal-associated domain-containing protein	245749_at	4.40	1.83E-04
AT4G30650	hydrophobic protein, putative / low temperature and salt responsive protein, putative	253627_at	4.30	4.47E-05
AT5G06690	(THIOREDOXIN-LIKE 5); thiol-disulfide exchange intermediate	250649_at	4.23	1.53E-02
AT1G67970	AT-HSFA8 (*Arabidopsis thaliana* heat shock transcription factor A8); DNA binding / transcription factor	259992_at	4.19	6.86E-04
AT1G79440	ALDH5F1 (SUCCINIC SEMIALDEHYDE DEHYDROGENASE); 3-chloroallyl aldehyde dehydrogenase/ succinate-semialdehyde dehydrogenase	262892_at	4.16	3.08E-03
AT5G11150	ATVAMP713 (*Arabidopsis thaliana* vesicle-associated membrane protein 713)	250412_at	3.99	1.60E-03
AT5G57110	ACA8 (AUTOINHIBITED CA2+ -ATPASE, ISOFORM 8); calcium-transporting ATPase/ calmodulin binding	247937_at	3.95	9.64E-03
AT2G19450	TAG1 (TRIACYLGLYCEROL BIOSYNTHESIS DEFECT 1); diacylglycerol O-acyltransferase	267280_at	3.95	1.67E-03
AT4G26670	mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein	253981_at	3.94	6.03E-05
AT1G29395	COR414-TM1 (cold regulated 414 thylakoid membrane 1)	259789_at	3.87	3.96E-03
AT1G70420	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT1G23710.1); similar to Protein of unknown function DUF1645 [Medicago truncatula] (GB:ABE93113.1); contains InterPro domain Protein of unknown function DUF1645; (InterPro:IPR012442)	264314_at	3.86	8.82E-04
AT2G40750	WRKY54 (WRKY DNA-binding protein 54); transcription factor	257382_at	3.76	1.09E-03
AT5G26340	MSS1 (SUGAR TRANSPORT PROTEIN 13); carbohydrate transporter/ hexose:hydrogen symporter/ high-affinity hydrogen:glucose transporter/ sugar porter	246831_at	3.70	3.46E-05
AT2G38465	unknown protein	267036_at	3.66	8.57E-03
AT2G28900	OEP16 (OUTER ENVELOPE PROTEIN 16); protein translocase	266225_at	3.60	1.35E-03
AT5G35735	auxin-responsive family protein	249719_at	3.58	4.03E-04
AT3G55450	protein kinase, putative	251789_at	3.53	1.14E-03
AT5G57630	CIPK21 (CBL-INTERACTING PROTEIN KINASE 21); kinase	247867_at	3.48	1.93E-02
AT5G39410	Identical to Probable mitochondrial saccharopine dehydrogenase At5g39410 (EC 1.5.1.9) (SDH) [*Arabidopsis Thaliana*]	249456_at	3.47	2.07E-04
AT4G25480	DREB1A (DEHYDRATION RESPONSE ELEMENT B1A); DNA binding / transcription factor/ transcriptional activator	254066_at	3.47	1.88E-03
AT1G49230	zinc finger (C3HC4-type RING finger) family protein	260753_at	3.45	6.49E-05
AT5G61380	TOC1 (TIMING OF CAB1 1); transcription regulator	247525_at	3.43	2.74E-02
AT1G22770	GI (GIGANTEA)	264211_at	3.42	7.49E-04
AT3G56710	SIB1 (SIGMA FACTOR BINDING PROTEIN 1); binding	246293_at	3.28	8.14E-03
AT1G53035	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT3G15358.1); similar to unknown [Musa acuminata] (GB:ABC41688.1)	261318_at	3.27	2.25E-03
AT1G48330	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT3G17580.1)	262236_at	3.27	3.53E-03
AT5G54960	PDC2 (PYRUVATE DECARBOXYLASE-2); pyruvate decarboxylase	248138_at	3.26	4.80E-04
AT4G32340	binding	253421_at	3.25	1.08E-03
AT5G15960; AT5G15970	[AT5G15960, KIN1];[AT5G15970, KIN2 (COLD-RESPONSIVE 6.6)]	246481_s_at	3.25	4.86E-03
AT1G20030	pathogenesis-related thaumatin family protein	261248_at	3.20	4.23E-08
AT2G02100	LCR69/PDF2.2 (Low-molecular-weight cysteine-rich 69); protease inhibitor	266119_at	3.15	3.01E-04
AT1G30040	ATGA2OX2; gibberellin 2-beta-dioxygenase	260023_at	3.12	9.47E-04
AT4G01130	acetylesterase, putative	255607_at	3.11	1.92E-04
AT4G19120	ERD3 (EARLY-RESPONSIVE TO DEHYDRATION 3)	254563_at	3.08	6.15E-04
AT5G03350	legume lectin family protein	250942_at	3.07	1.46E-03
AT4G39260	ATGRP8/GR-RBP8 (COLD, CIRCADIAN RHYTHM, AND RNA BINDING 1, GLYCINE-RICH PROTEIN 8)	252885_at	3.06	1.37E-04

Supplementary Table S5

Locus	Description	Probeset	Fold Change	P value
AT4G09020	ATISA3/ISA3 (ISOAMYLASE 3); alpha-amylase	255070_at	2.99	1.51E-04
AT3G51660	macrophage migration inhibitory factor family protein / MIF family protein	252076_at	2.97	6.01E-05
AT5G26570	PWD (PHOSPHOGLUCAN WATER DIKINASE); catalytic	246829_at	2.96	2.04E-04
AT5G20630	GLP3 (GERMIN-LIKE PROTEIN 3); manganese ion binding / metal ion binding / nutrient reservoir	246004_at	2.93	5.08E-05
AT2G25930	ELF3 (EARLY FLOWERING 3)	266839_at	2.81	1.53E-03
AT1G19960	similar to transmembrane receptor [*Arabidopsis thaliana*] (TAIR:AT2G32140.1)	261221_at	2.79	2.04E-04
AT3G47160	protein binding / zinc ion binding	252464_at	2.76	9.68E-04
AT3G05880	RCI2A (RARE-COLD-INDUCIBLE 2A)	258735_at	2.74	1.68E-03
AT4G29610	cytidine deaminase, putative / cytidine aminohydrolase, putative	253679_at	2.73	4.69E-05
AT4G34950	nodulin family protein	253215_at	2.72	1.95E-02
AT1G09350	ATGOLS3 (*ARABIDOPSIS THALIANA* GALACTINOL SYNTHASE 3); transferase, transferring glycosyl groups / transferase, transferring hexosyl groups	264511_at	2.71	4.03E-03
AT1G76790	O-methyltransferase family 2 protein	259878_at	2.70	1.14E-02
AT1G26665	similar to RNA polymerase II mediator complex protein-related [*Arabidopsis thaliana*] (TAIR:AT5G41910.1); similar to At1g26660/T24P13_4 [Medicago truncatula] (GB:ABE78676.1); similar to Os09g0528300 [Oryza sativa (japonica cultivar-group)] (GB:NP_001063737	261272_at	2.68	5.18E-03
AT1G76590	zinc-binding family protein	259977_at	2.67	2.91E-03
AT3G46970	ATPHS2/PHS2 (ALPHA-GLUCAN PHOSPHORYLASE 2); phosphorylase/ transferase, transferring glycosyl groups	252468_at	2.67	1.72E-05
AT3G63160	similar to outer envelope membrane protein, putative [*Arabidopsis thaliana*] (TAIR:AT3G52420.1); similar to chloroplast outer envelope membrane protein [Erysimum cheiri] (GB:AAK52964.1)	251155_at	2.66	1.18E-04
AT4G39090	RD19 (RESPONSIVE TO DEHYDRATION 19); cysteine-type peptidase	252927_at	2.64	7.51E-03
AT1G51610	cation efflux family protein / metal tolerance protein, putative (MTPc4)	260489_at	2.63	3.72E-05
AT5G63810	BGAL10 (beta-galactosidase 10); beta-galactosidase	247348_at	2.63	1.55E-02
AT5G25210	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT4G32030.1)	246929_at	2.63	1.08E-02
AT1G22570	proton-dependent oligopeptide transport (POT) family protein	261937_at	2.59	1.48E-02
AT1G31680	copper amine oxidase family protein	246573_at	2.58	6.28E-03
AT1G06460	ACD32.1 (ALPHA-CRYSTALLIN DOMAIN 31.2)	262629_at	2.57	3.96E-05
AT1G80480	PTAC17 (PLASTID TRANSCRIPTIONALLY ACTIVE17)	260283_at	2.56	4.41E-05
AT5G47240	ATNUDT8 (*Arabidopsis thaliana* Nudix hydrolase homolog 8); hydrolase	248793_at	2.55	5.99E-04
AT1G27630	cyclin family protein	262296_at	2.55	6.93E-04
AT5G23410; AT1G68050; AT5G42730	[AT5G23410, similar to FKF1 (FLAVIN-BINDING KELCH DOMAIN F BOX PROTEIN), ubiquitin-protein ligase [*Arabidopsis thaliana*] (TAIR:AT1G68050.1); similar to Cyclin-like F-box; Galactose oxidase, central [Medicago truncatula] (GB:ABE90708.1); contains InterPro	259990_s_at	2.54	2.25E-02
AT3G47800	aldose 1-epimerase family protein	252387_at	2.53	7.73E-03
AT1G12710	ATPP2-A12 (Phloem protein 2-A12)	255931_at	2.53	2.46E-02
AT4G33490	pepsin A	253331_at	2.52	5.01E-03
AT3G28290; AT3G28300	[AT3G28290, AT14A];[AT3G28300, AT14A]	256601_s_at	2.51	2.56E-02
AT1G10760	SEX1 (STARCH EXCESS 1)	262784_at	2.50	3.83E-05
AT3G47860	apolipoprotein D-related	252391_at	2.49	2.04E-03
AT1G75960	AMP-binding protein, putative	262698_at	2.45	5.79E-04
AT1G49720	ABF1 (ABSCISIC ACID RESPONSIVE ELEMENT-BINDING FACTOR 1); DNA binding / transcription factor/ transcriptional activator	261613_at	2.44	3.67E-03
AT1G53885	senescence-associated protein-related	262226_at	2.42	1.01E-02
AT2G17840	ERD7 (EARLY-RESPONSIVE TO DEHYDRATION 7)	264787_at	2.39	3.60E-03
AT1G28050	zinc finger (B-box type) family protein	259595_at	2.38	4.47E-03
AT5G14920	gibberellin-regulated family protein	246550_at	2.35	4.79E-02
AT3G10410	SCPL49 (serine carboxypeptidase-like 49); serine carboxypeptidase	258970_at	2.33	3.41E-03
AT5G50680; AT5G50580	[AT5G50680, SUMO activating enzyme 1b (SAE1b)];[AT5G50580, SAE1B (SUMO-ACTIVATING ENZYME 1B); SUMO activating enzyme]	248523_s_at	2.31	3.14E-09
AT2G06925	ATSPLA2-ALPHA/PLA2-ALPHA (PHOSPHOLIPASE A2-ALPHA); phospholipase A2	266500_at	2.30	1.40E-04
AT2G29630	thiamine biosynthesis family protein / thiC family protein	266673_at	2.30	2.92E-04
AT4G14270	Protein containing PAM2 motif which mediates interaction with the PABC domain of polyadenyl binding proteins.	245602_at	2.29	1.64E-03
AT5G60540	ATPDX2/EMB2407/PDX2 (PYRIDOXINE BIOSYNTHESIS 2); glutaminase/ glutamyl-tRNA(Gln) amidotransferase/ protein heterodimerization	247641_at	2.27	2.20E-04
AT3G18080	glycosyl hydrolase family 1 protein	258151_at	2.27	1.22E-07
AT2G45560	CYP76C1 (cytochrome P450, family 76, subfamily C, polypeptide 1); heme binding / iron ion binding / monooxygenase	267505_at	2.27	1.19E-06
AT3G26740	CCL (CCR-LIKE)	257832_at	2.27	1.85E-02
AT4G35480	RHA3B (RING-H2 finger A3B); protein binding / zinc ion binding	253140_at	2.26	1.72E-05
AT1G12730	cell division cycle protein-related	255939_at	2.25	3.31E-03
AT1G48210	serine/threonine protein kinase, putative	260728_at	2.24	7.95E-03
AT4G12290; AT4G12280	[AT4G12290, copper amine oxidase, putative];[AT4G12280, copper amine oxidase family protein]	254833_s_at	2.24	2.28E-02
AT1G68500	unknown protein	260264_at	2.23	1.86E-04
AT5G14550	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT1G62305.1); similar to Os01g0695200 [Oryza sativa (japonica cultivar-group)] (GB:NP_001043958.1); similar to Protein of unknown function DUF266, plant [Medicago truncatula] (GB:ABD28621.1); similar	250194_at	2.22	1.57E-02
AT1G17460	TRFL3 (TRF-LIKE 3); DNA binding / transcription factor	261086_at	2.20	4.81E-04
AT1G31850	dehydration-responsive protein, putative	246288_at	2.20	1.35E-03
AT4G11360	RHA1B (RING-H2 finger A1B); protein binding / zinc ion binding	254919_at	2.20	4.63E-03
AT2G47890	zinc finger (B-box type) family protein	266514_at	2.19	3.42E-03
AT5G62720	integral membrane HPP family protein	247443_at	2.18	1.35E-03
AT4G14230	CBS domain-containing protein-related	245600_at	2.17	1.68E-04
AT5G03240	UBQ3 (POLYUBIQUITIN 3); protein binding	250935_at	2.17	8.38E-04
AT2G28840	ankyrin repeat family protein	266229_at	2.17	6.75E-03
AT4G33700	CBS domain-containing protein	253351_at	2.15	4.01E-04
AT1G21680	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT1G21670.1); similar to WD40 domain protein beta Propeller [Solibacter usitatus Ellin6076]	262505_at	2.15	6.65E-03

Locus	Description	Probeset	Fold Change	P value
AT5G08260	SCPL35 (serine carboxypeptidase-like 35); serine carboxypeptidase	250517_at	2.15	1.83E-04
AT2G31360	ADS2 (16:0DELTA9 *ARABIDOPSIS* DESATURASE 2); oxidoreductase	263249_at	2.14	6.93E-04
AT1G64890	integral membrane transporter family protein	262881_at	2.14	3.61E-04
AT4G27130	eukaryotic translation initiation factor SUI1, putative	253900_at	2.14	1.60E-02
AT3G53460	CP29 (chloroplast 29 kDa ribonucleoprotein); RNA binding	251956_at	2.14	1.61E-04
AT2G42540	COR15A (COLD-REGULATED 15A)	263497_at	2.13	3.54E-02
AT5G54930	AT hook motif-containing protein	248148_at	2.11	2.92E-04
AT2G36390	SBE2.1 (STARCH BRANCHING ENZYME 2.1); 1,4-alpha-glucan branching enzyme	263912_at	2.10	1.83E-03
AT3G53800	armadillo/beta-catenin repeat family protein	251919_at	2.10	1.87E-03
AT4G02370	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT1G02816.1); similar to Os05g0362300 [Oryza sativa (japonica cultivar-group)] (GB:NP_001055314.1); similar to Protein of unknown function, DUF538 [Oryza sativa (japonica cultivar-group)] (GB:AAX9540	255477_at	2.10	3.36E-03
AT2G43550	trypsin inhibitor, putative	260547_at	2.09	3.96E-05
AT2G25730	binding / heme binding	265900_at	2.07	1.10E-02
AT3G51400	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT4G35720.1); similar to hypothetical protein [Glycine max] (GB:AAK01735.1); contains InterPro domain Protein of unknown function DUF241, plant; (InterPro:IPR004320)	252118_at	2.07	1.70E-05
AT3G58570	DEAD box RNA helicase, putative	251529_at	2.06	2.66E-04
AT1G75190	similar to GTP binding / RNA binding [*Arabidopsis thaliana*] (TAIR:AT4G26630.2); similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT2G42190.1); similar to PREDICTED: hypothetical protein [Mus musculus] (GB:XP_001004948.1)	256455_at	2.05	4.07E-03
AT1G11530	ATCXXS1 (C-TERMINAL CYSTEINE RESIDUE IS CHANGED TO A SERINE 1); thiol-disulfide exchange intermediate	261821_at	2.05	6.57E-03
AT5G63420	EMB2746 (EMBRYO DEFECTIVE 2746); catalytic	247385_at	2.05	2.07E-04
AT5G02860	pentatricopeptide (PPR) repeat-containing protein	250987_at	2.05	9.68E-04
AT1G13270	MAP1C (METHIONINE AMINOPEPTIDASE 1B); metalloexopeptidase	259363_at	2.04	1.65E-04
AT1G34380	5'-3' exonuclease family protein	259928_at	2.04	1.59E-05
AT1G59870	PDR8/PEN3 (PLEIOTROPIC DRUG RESISTANCE8); ATPase, coupled to transmembrane movement of substances	262899_at	2.04	1.38E-02
AT1G67660	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT1G13810.1); similar to unknown protein [Oryza sativa (japonica cultivar-group)] (GB:BAD05466.1); contains domain no description (G3D.3.90.320.10); contains domain ALPHA/BETA HYDROLASE RELATED (PTHR	245188_at	2.03	3.00E-04
AT4G17120	similar to C2 domain-containing protein [*Arabidopsis thaliana*] (TAIR:AT1G48090.2); similar to unknown protein [Oryza sativa] (GB:AAG60185.1); similar to Os10g0565300 [Oryza sativa (japonica cultivar-group)] (GB:NP_001065419.1); contains InterPro domain C2	245434_at	2.03	2.30E-02
AT1G12845	similar to hypothetical protein MtrDRAFT_AC149131g9v1 [Medicago truncatula] (GB:ABD32556.1)	261203_at	2.02	6.47E-06
AT4G27440	PORB (PROTOCHLOROPHYLLIDE OXIDOREDUCTASE B); oxidoreductase/ protochlorophyllide reductase	253871_at	2.02	1.46E-03
AT5G58600	PMR5 (POWDERY MILDEW RESISTANT 5)	247786_at	2.02	7.86E-03
AT4G31050	lipoyltransferase (LIP2p)	253553_at	2.01	6.44E-04
AT5G64860	DPE1 (DISPROPORTIONATING ENZYME); 4-alpha-glucanotransferase	247216_at	2.00	1.83E-04
DOWNREGULATED GENES				
AT2G33850	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT1G28400.1); similar to unknown [Brassica napus] (GB:AAC06020.1)	267459_at	-66.84	7.91E-19
AT5G33370	GDSL-motif lipase/hydrolase family protein	246687_at	-19.38	5.25E-14
AT3G30720	unknown protein	256940_at	-11.58	5.80E-13
AT1G01600	CYP86A4 (cytochrome P450, family 86, subfamily A, polypeptide 4); oxygen binding	259429_at	-7.62	1.80E-09
AT2G04032	ZIP7 (ZINC TRANSPORTER 7 PRECURSOR); cation transporter	263480_at	-7.12	5.58E-09
AT1G06100	fatty acid desaturase family protein	260948_at	-6.92	2.53E-09
AT2G21140	ATPRP2 (PROLINE-RICH PROTEIN 2)	264007_at	-6.35	4.08E-13
AT3G59010	pectinesterase family protein	251509_at	-5.64	4.05E-11
AT1G01060	LHY (LATE ELONGATED HYPOCOTYL); DNA binding / transcription factor	261569_at	-5.29	7.09E-05
AT1G79840	GL2 (GLABRA 2); DNA binding / transcription factor	260166_at	-5.03	1.87E-11
AT1G63710	CYP86A7 (cytochrome P450, family 86, subfamily A, polypeptide 7); oxygen binding	260241_at	-4.89	8.58E-09
AT5G40330	MYB23 (myb domain protein 23); DNA binding / transcription factor	249408_at	-4.75	1.05E-09
AT5G01600	ATFER1 (ferretin 1); ferric iron binding	251109_at	-4.64	1.80E-03
AT1G65450	transferase family protein	264160_at	-4.39	1.53E-08
AT1G22890	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT5G44568.1); contains domain FAMILY NOT NAMED (PTHR12953); contains domain SUBFAMILY NOT NAMED (PTHR12953:SF10)	264774_at	-4.29	9.89E-04
AT5G13170	nodulin MtN3 family protein	245982_at	-4.21	1.49E-03
AT5G10430	AGP4 (ARABINOGALACTAN-PROTEIN 4)	250437_at	-4.17	7.14E-07
AT5G48850	male sterility MS5 family protein	248676_at	-4.17	2.31E-04
AT2G27420	cysteine proteinase, putative	265665_at	-4.17	3.83E-03
AT1G02820	late embryogenesis abundant 3 family protein / LEA3 family protein	262113_at	-4.16	1.78E-03
AT4G11650	ATOSM34 (OSMOTIN 34)	254889_at	-3.95	3.12E-02
AT2G46830	CCA1 (CIRCADIAN CLOCK ASSOCIATED 1); transcription factor	266719_at	-3.89	3.94E-02
AT1G27760	interferon-related developmental regulator family protein / IFRD protein family	261651_at	-3.60	5.73E-06
AT5G58770	dehydrodolichyl diphosphate synthase, putative / DEDOL-PP synthase, putative	247780_at	-3.51	1.07E-03
AT3G09600	myb family transcription factor	258723_at	-3.42	7.40E-03
AT5G52570	BETA-OHASE 2 (BETA-CAROTENE HYDROXYLASE 2); beta-carotene hydroxylase	248311_at	-3.33	1.24E-02
AT1G65445	transferase-related	264163_at	-3.32	1.12E-05
AT3G12580	HSP70 (heat shock protein 70); ATP binding	256245_at	-3.27	1.13E-03
AT1G65490	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT1G65500.1)	264636_at	-3.26	3.78E-02
AT3G08860	alanine--glyoxylate aminotransferase, putative / beta-alanine-pyruvate aminotransferase, putative / AGT, putative	258983_at	-3.26	1.35E-04
AT2G47180	ATGOLS1 (*ARABIDOPSIS THALIANA* GALACTINOL SYNTHASE 1); transferase, transferring hexosyl groups	263320_at	-3.23	8.47E-06
AT1G32900	starch synthase, putative	261191_at	-3.21	3.47E-02
AT1G10370	ATGSTU17/ERD9/GST30/GST30B (EARLY-RESPONSIVE TO DEHYDRATION 9, GLUTATHIONE S-TRANSFERASE 30, GLUTATHIONE S-TRANSFERASE 30B); glutathione transferase	264436_at	-3.21	6.13E-04
AT3G12320	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT5G06980.1); similar to ACI112 [Lycopersicon esculentum] (GB:AAY97870.1)	256266_at	-3.17	4.89E-02

Locus	Description	Probeset	Fold Change	P value
AT4G28160	hydroxyproline-rich glycoprotein family protein	253800_at	-3.16	1.12E-05
AT4G30290	ATXTH19 (XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 19); hydrolase, acting on glycosyl bonds	253608_at	-3.16	1.98E-02
AT3G17609	HYH (HY5-HOMOLOG); DNA binding / transcription factor	258349_at	-3.01	4.55E-03
AT5G14760	AO (L-ASPARTATE OXIDASE); L-aspartate oxidase	246597_at	-2.97	5.79E-04
AT5G55720	pectate lyase family protein	248073_at	-2.91	8.06E-06
AT1G25450	very-long-chain fatty acid condensing enzyme, putative	255732_at	-2.88	2.61E-08
AT3G27170	CLC-B (chloride channel protein B); anion channel/ voltage-gated chloride channel	256751_at	-2.87	1.44E-03
AT1G69490	NAP (NAC-LIKE, ACTIVATED BY AP3/PI); transcription factor	256300_at	-2.85	3.72E-02
AT4G33550	lipid binding	253344_at	-2.80	6.47E-03
AT3G10570	CYP77A6 (cytochrome P450, family 77, subfamily A, polypeptide 6); oxygen binding	258962_at	-2.79	2.23E-06
AT2G34660	ATMRP2 (MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN 2); ATPase, coupled to transmembrane movement of substances	267319_at	-2.79	7.17E-06
AT2G22240	inositol-3-phosphate synthase isozyme 2 / myo-inositol-1-phosphate synthase 2 / MI-1-P synthase 2 / IPS 2	263433_at	-2.77	1.68E-03
AT2G37870	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	266098_at	-2.76	3.99E-04
AT2G33380	RD20 (RESPONSIVE TO DESSICATION 20); calcium ion binding	255795_at	-2.75	5.16E-03
AT3G28270	similar to AT14A [*Arabidopsis thaliana*] (TAIR:AT3G28290.1); similar to AT14A [*Arabidopsis thaliana*] (TAIR:AT3G28300.1); similar to Protein of unknown function DUF677 [Medicago truncatula] (GB:ABE78510.1); contains InterPro domain Protein of unknown functi	256603_at	-2.68	2.68E-03
AT2G41250	haloacid dehalogenase-like hydrolase family protein	266363_at	-2.68	2.56E-02
AT1G80760	NIP6;1 (NOD26-like intrinsic protein 6;1); water channel	261881_at	-2.62	1.35E-03
AT5G06980	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT3G12320.1)	250665_at	-2.61	2.62E-02
AT3G24460	TMS membrane family protein / tumour differentially expressed (TDE) family protein	256619_at	-2.61	1.71E-04
AT2G37760	aldo/keto reductase family protein	267181_at	-2.59	2.04E-04
AT3G51240	F3H (TRANSPARENT TESTA 6); naringenin 3-dioxygenase	252123_at	-2.56	1.13E-02
AT3G54500	similar to dentin sialophosphoprotein-related [*Arabidopsis thaliana*] (TAIR:AT5G64170.2); similar to conserved hypothetical protein [Medicago truncatula] (GB:ABD28297.1)	251869_at	-2.54	1.10E-02
AT1G12570	glucose-methanol-choline (GMC) oxidoreductase family protein	259526_at	-2.52	4.57E-06
AT4G01080	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT1G01430.1); similar to unknown protein Cr17 [Brassica napus] (GB:AAX51387.1); contains InterPro domain Protein of unknown function DUF231, plant; (InterPro:IPR004253)	255604_at	-2.49	3.49E-03
AT1G64780	ATAMT1;2 (AMMONIUM TRANSPORTER 1;2); ammonium transporter	262883_at	-2.48	1.67E-02
AT1G62540	flavin-containing monooxygenase family protein / FMO family protein	265122_at	-2.48	1.35E-03
AT4G08300	nodulin MtN21 family protein	255127_at	-2.48	4.50E-03
AT2G20870	cell wall protein precursor, putative	265441_at	-2.47	7.31E-07
AT5G24120	SIGE (RNA polymerase sigma subunit E); DNA binding / DNA-directed RNA polymerase/ sigma factor/ transcription factor	249769_at	-2.47	3.94E-02
AT5G22460	esterase/lipase/thioesterase family protein	249917_at	-2.45	8.10E-04
AT5G04660	CYP77A4 (cytochrome P450, family 77, subfamily A, polypeptide 4); oxygen binding	250859_at	-2.43	3.18E-05
AT3G48460	GDSL-motif lipase/hydrolase family protein	252363_at	-2.43	1.35E-03
AT1G62510	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	265111_at	-2.42	8.31E-03
AT5G51720	similar to zinc finger, CDGSH-type domain 2 [Homo sapiens] (GB:NP_001008389.1); similar to Os07g0467200 [Oryza sativa (japonica cultivar-group)] (GB:NP_001059590.1); similar to hypothetical protein [Homo sapiens] (GB:CAD97935.1); contains InterPro domain	248377_at	-2.39	3.59E-02
AT5G45360	F-box family protein	248966_at	-2.36	1.71E-09
AT2G37770	aldo/keto reductase family protein	267168_at	-2.36	6.57E-03
AT3G10340	phenylalanine ammonia-lyase, putative	259149_at	-2.35	8.06E-06
AT1G55960	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT3G13062.1); similar to Lipid-binding START [Medicago truncatula] (GB:ABE91086.1); contains InterPro domain Lipid-binding START; (InterPro:IPR002913)	260603_at	-2.34	9.11E-03
AT4G14690	ELIP2 (EARLY LIGHT-INDUCIBLE PROTEIN 2); chlorophyll binding	245306_at	-2.32	5.75E-03
AT5G01740	similar to wound-responsive protein-related [*Arabidopsis thaliana*] (TAIR:AT3G10985.1); similar to Wound-induced protein WI12, putative [Medicago truncatula] (GB:ABE88200.1); contains InterPro domain Wound-induced WI12; (InterPro:IPR009798)	251072_at	-2.32	3.21E-03
AT3G01140	MYB106 (myb domain protein 106); DNA binding / transcription factor	259281_at	-2.32	1.09E-03
AT5G23730	nucleotide binding	249798_at	-2.32	3.01E-04
AT5G15850	COL1 (CONSTANS-LIKE 1); transcription factor/ zinc ion binding	246523_at	-2.31	4.50E-02
AT5G23940	EMB3009 (EMBRYO DEFECTIVE 3009); transferase	249813_at	-2.31	5.93E-04
AT5G12420	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT5G16350.1); similar to Protein of unknown function DUF1298 [Medicago truncatula] (GB:ABE82755.1); contains InterPro domain Protein of unknown function UPF0089; (InterPro:IPR004255); contains InterP	245181_at	-2.30	2.61E-04
AT3G56200	amino acid transporter family protein	251722_at	-2.27	1.10E-02
AT3G51895	SULTR3;1 (SULFATE TRANSPORTER 1); sulfate transporter	246310_at	-2.25	9.41E-03
AT2G46140	late embryogenesis abundant protein, putative / LEA protein, putative	266581_at	-2.24	1.59E-05
AT5G44050	MATE efflux family protein	249071_at	-2.22	2.55E-03
AT4G30470	cinnamoyl-CoA reductase-related	253638_at	-2.21	3.18E-05
AT1G79270	ECT8 (evolutionarily conserved C-terminal region 8)	264102_at	-2.20	1.57E-02
AT1G07180	NDA1 (ALTERNATIVE NAD(P)H DEHYDROGENASE 1); NADH dehydrogenase	256057_at	-2.19	2.09E-02
AT5G06530	ABC transporter family protein	250690_at	-2.18	2.51E-03
AT4G31870	ATGPX7 (GLUTATHIONE PEROXIDASE 7); glutathione peroxidase	253496_at	-2.18	2.69E-03
AT5G08030	glycerophosphoryl diester phosphodiesterase family protein	250561_at	-2.16	2.23E-02
AT3G18170	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT3G18180.1); similar to glycosyltransferase [Saccharum officinarum] (GB:CAI30073.1); contains InterPro domain Protein of unknown function DUF563; (InterPro:IPR007657)	258143_at	-2.13	2.49E-04
AT2G32160	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT2G32170.1); similar to Os05g0511300 [Oryza sativa (japonica cultivar-group)] (GB:NP_001056014.1); similar to unnamed protein product [Tetraodon nigroviridis] (GB:CAF92601.1); contains InterPro doma	265698_at	-2.12	1.57E-02
AT1G58290	HEMA1; glutamyl-tRNA reductase	256020_at	-2.12	1.73E-02
AT4G15530	PPDK (PYRUVATE ORTHOPHOSPHATE DIKINASE); kinase/ pyruvate, phosphate dikinase	245528_at	-2.12	6.63E-03
AT3G61840	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT2G46535.1)	251284_at	-2.11	8.47E-06
AT1G17050	SPS2 (Solanesyl diphosphate synthase 2); dimethylallyltranstransferase	262526_at	-2.11	5.88E-03
AT5G54130	calcium ion binding	248191_at	-2.09	3.59E-02
AT3G24170	ATGR1; glutathione-disulfide reductase	257252_at	-2.08	2.27E-03

Locus	Description	Probeset	Fold Change	P value
AT4G35320	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT2G17300.1); similar to Os02g0715300 [Oryza sativa (japonica cultivar-group)] (GB:NP_001047925.1); similar to Os08g0511400 [Oryza sativa (japonica cultivar-group)] (GB:NP_001062213.1); contains doma	253165_at	-2.08	2.53E-03
AT3G56290	similar to Os01g0823600 [Oryza sativa (japonica cultivar-group)] (GB:NP_001044661.1); similar to unnamed protein product [Ostreococcus tauri] (GB:CAL58546.1)	251727_at	-2.07	9.10E-03
AT1G27940	PGP13 (P-GLYCOPROTEIN 13); ATPase, coupled to transmembrane movement of substances	259607_at	-2.05	6.13E-04
AT1G62180	APR2 (5'ADENYLYLPHOSPHOSULFATE REDUCTASE 2)	264745_at	-2.02	6.63E-03
AT5G58120	disease resistance protein (TIR-NBS-LRR class), putative	247848_at	-2.02	3.68E-02
AT3G18560	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT1G49000.1); similar to Os02g0711400 [Oryza sativa (japonica cultivar-group)] (GB:NP_001047903.1); similar to hypothetical protein [Oryza sativa (japonica cultivar-group)] (GB:BAD45365.1)	256799_at	-2.01	2.11E-02
AT5G44130	fasciclin-like arabinogalactan-protein, putative	249037_at	-2.01	3.03E-02
AT1G71440	PFI (PFIFFERLING); protein binding	259895_at	-2.01	9.94E-09
AT1G29050	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT2G34070.1); similar to unknown [Pisum sativum] (GB:ABA29158.1); contains InterPro domain Protein of unknown function DUF231, plant; (InterPro:IPR004253)	260840_at	-2.01	3.95E-04
AT2G04795	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT5G35732.1)	263632_at	-2.01	1.01E-02

Supplementary Table S6. GOLS2-dependent genes

Locus	Description	Probeset	Fold Change	P value
UPREGULATED GENES				
AT1G56600	ATGOLS2 (*ARABIDOPSIS THALIANA* GALACTINOL SYNTHASE 2); transferase, transferring glycosyl groups / transferase, transferring hexosyl groups	245627_at	109.17	3.55E-14
AT2G19800	MIOX2 (MYO-INOSITOL OXYGENASE 2)	266693_at	54.13	6.40E-04
AT3G22231	PCC1 (PATHOGEN AND CIRCADIAN CONTROLLED 1)	256766_at	13.66	8.83E-05
AT5G23240; AT5G23235	[AT5G23240, DNAJ heat shock N-terminal domain-containing protein]	249850_at	9.70	1.17E-02
AT5G36230	eIF4-gamma/eIF5/eIF2-epsilon domain-containing protein	246621_at	7.99	1.13E-15
AT5G62360	invertase/pectin methylesterase inhibitor family protein	247478_at	7.55	2.79E-03
AT5G24470	APRR5 (PSEUDO-RESPONSE REGULATOR 5); transcription regulator	249741_at	6.02	1.09E-03
AT3G22240	unknown protein	256617_at	5.84	1.18E-04
AT1G56300	DNAJ heat shock N-terminal domain-containing protein	256221_at	5.46	2.03E-02
AT2G39920	acid phosphatase class B family protein	267361_at	5.14	4.01E-05
AT4G04330	similar to unnamed protein product [Ostreococcus tauri] (GB:CAL56420.1); similar to Os08g0425200 [Oryza sativa (japonica cultivar-group)] (GB:NP_001061837.1)	255331_at	4.49	1.40E-02
AT1G22570	proton-dependent oligopeptide transport (POT) family protein	261937_at	3.98	1.85E-03
AT1G03850	glutaredoxin family protein	265067_at	3.90	1.29E-02
AT5G03350	legume lectin family protein	250942_at	3.71	9.82E-04
AT2G21130	peptidyl-prolyl cis-trans isomerase / cyclophilin (CYP2) / rotamase	264019_at	3.71	1.01E-02
AT5G26340	MSS1 (SUGAR TRANSPORT PROTEIN 13); carbohydrate transporter/ hexose:hydrogen symporter/ high-affinity hydrogen:glucose transporter/ sugar porter	246831_at	3.63	1.60E-04
AT5G35735	auxin-responsive family protein	249719_at	3.58	1.19E-03
AT4G16146	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT1G69510.2); similar to negatively light-regulated protein, putative, expressed [Oryza sativa (japonica cultivar-group)] (GB:ABA97694.2);	245319_at	3.39	1.31E-02
AT1G33730; AT1G33720	[AT1G33730, CYP76C5 (cytochrome P450, family 76, subfamily C, polypeptide 5); oxygen binding];[AT1G33720, CYP76C6 (cytochrome P450, family 76, subfamily C, polypeptide 6); oxygen binding]	261986_s_at	3.25	6.59E-03
AT3G56710	SIB1 (SIGMA FACTOR BINDING PROTEIN 1); binding	246293_at	3.09	2.09E-02
AT1G67970	AT-HSFA8 (*Arabidopsis thaliana* heat shock transcription factor A8); DNA binding / transcription factor	259992_at	3.08	1.17E-02
AT1G65330; AT1G65300	[AT1G65330, PHE1 (PHERES1); DNA binding / transcription factor];[AT1G65300, PHE2 (PHERES2); DNA binding / transcription factor]	264214_s_at	3.06	4.22E-04
AT1G51090	heavy-metal-associated domain-containing protein	245749_at	2.93	8.71E-03
AT1G79440	ALDH5F1 (SUCCINIC SEMIALDEHYDE DEHYDROGENASE); 3-chloroallyl aldehyde dehydrogenase/ succinate-semialdehyde dehydrogenase	262892_at	2.91	4.03E-02
AT1G11210	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT1G11220.1); similar to fiber expressed protein [Gossypium hirsutum] (GB:AAY85179.1); similar to cotton fiber expressed protein 1 [Gossypium hirsutum] (GB:AAC33276.1); contains InterPro domain Prote	262452_at	2.91	2.79E-03
AT1G20030	pathogenesis-related thaumatin family protein	261248_at	2.91	7.11E-07
AT4G29610	cytidine deaminase, putative / cytidine aminohydrolase, putative	253679_at	2.90	8.83E-05
AT4G26670	mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein	253981_at	2.69	4.26E-03
AT1G09350	ATGOLS3 (*ARABIDOPSIS THALIANA* GALACTINOL SYNTHASE 3); transferase, transferring glycosyl groups / transferase, transferring hexosyl groups	264511_at	2.68	9.79E-03
AT1G53885	senescence-associated protein-related	262226_at	2.60	1.17E-02
AT3G47160	protein binding / zinc ion binding	252464_at	2.57	4.64E-03
AT2G19450	TAG1 (TRIACYLGLYCEROL BIOSYNTHESIS DEFECT 1); diacylglycerol O-acyltransferase	267280_at	2.57	4.39E-02
AT2G40750	WRKY54 (WRKY DNA-binding protein 54); transcription factor	257382_at	2.54	2.98E-02
AT5G54960	PDC2 (PYRUVATE DECARBOXYLASE-2); pyruvate decarboxylase	248138_at	2.51	1.01E-02
AT4G30650	hydrophobic protein, putative / low temperature and salt responsive protein, putative	253627_at	2.48	1.06E-02
AT4G32340	binding	253421_at	2.45	1.89E-02
AT1G70420	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT1G23710.1); similar to Protein of unknown function DUF1645 [Medicago truncatula] (GB:ABE93113.1); contains InterPro domain Protein of unknown function DUF1645; (InterPro:IPR012442)	264314_at	2.44	3.73E-02
AT2G26560	PLP2 (PHOSPHOLIPASE A 2A); nutrient reservoir	245038_at	2.41	1.72E-02
AT5G47240	ATNUDT8 (*Arabidopsis thaliana* Nudix hydrolase homolog 8); hydrolase	248793_at	2.37	3.19E-03
AT5G25210	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT4G32030.1)	246929_at	2.36	3.88E-02
AT2G18050	HIS1-3 (HISTONE H1-3); DNA binding	265817_at	2.35	1.27E-02
AT4G39090	RD19 (RESPONSIVE TO DEHYDRATION 19); cysteine-type peptidase	252927_at	2.32	3.32E-02
AT5G50680; AT5G50580	[AT5G50680, SUMO activating enzyme 1b (SAE1b)];[AT5G50580, SAE1B (SUMO-ACTIVATING ENZYME 1B); SUMO activating enzyme]	248523_s_at	2.32	9.26E-09
AT1G75960	AMP-binding protein, putative	262698_at	2.30	2.79E-03
AT1G19960	similar to transmembrane receptor [*Arabidopsis thaliana*] (TAIR:AT2G32140.1)	261221_at	2.28	4.24E-03
AT1G52200	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT3G18470.1); similar to Uncharacterized Cys-rich domain [Medicago truncatula] (GB:ABD32291.1); contains InterPro domain Protein of unknown function Cys-rich; (InterPro:IPR006461)	259841_at	2.28	3.85E-02
AT1G22770	GI (GIGANTEA)	264211_at	2.24	3.47E-02
AT5G24530	oxidoreductase, 2OG-Fe(II) oxygenase family protein	249754_at	2.21	1.31E-02
AT1G51610	cation efflux family protein / metal tolerance protein, putative (MTPc4)	260489_at	2.19	1.09E-03
AT2G28840	ankyrin repeat family protein	266229_at	2.19	1.29E-02
AT3G47800	aldose 1-epimerase family protein	252387_at	2.19	4.01E-02
AT1G76590	zinc-binding family protein	259977_at	2.17	2.93E-02
AT4G00970	protein kinase family protein	255654_at	2.16	5.09E-03
AT3G14050	RSH2 (RELA-SPOT HOMOLOG); catalytic	258207_at	2.15	3.06E-02
AT1G49720	ABF1 (ABSCISIC ACID RESPONSIVE ELEMENT-BINDING FACTOR 1); DNA binding / transcription factor/ transcriptional activator	261613_at	2.14	2.11E-02
AT2G02100	LCR69/PDF2.2 (Low-molecular-weight cysteine-rich 69); protease inhibitor	266119_at	2.12	2.09E-02
AT4G20860	FAD-binding domain-containing protein	254447_at	2.11	2.82E-02
AT3G46970	ATPHS2/PHS2 (ALPHA-GLUCAN PHOSPHORYLASE 2); phosphorylase/ transferase, transferring glycosyl groups	252468_at	2.07	1.42E-03

Supplementary Table S6

Locus	Description	Probeset	Fold Change	P value
AT2G43535	trypsin inhibitor, putative	260549_at	2.07	2.04E-03
AT1G12730	cell division cycle protein-related	255939_at	2.07	1.59E-02
AT1G27630	cyclin family protein	262296_at	2.06	1.29E-02
AT3G63160	similar to outer envelope membrane protein, putative [*Arabidopsis thaliana*] (TAIR:AT3G52420.1); similar to chloroplast outer envelope membrane protein [Erysimum cheiri] (GB:AAK52964.1)	251155_at	2.06	5.31E-03
AT2G39570	ACT domain-containing protein	266984_at	2.06	4.82E-02
AT2G25930	ELF3 (EARLY FLOWERING 3)	266839_at	2.04	4.03E-02
AT3G51660	macrophage migration inhibitory factor family protein / MIF family protein	252076_at	2.03	9.28E-03
AT4G23270	protein kinase family protein	254248_at	2.02	4.67E-03
AT3G05880	RCI2A (RARE-COLD-INDUCIBLE 2A)	258735_at	2.02	4.13E-02
AT1G06460	ACD32.1 (ALPHA-CRYSTALLIN DOMAIN 31.2)	262629_at	2.01	2.67E-03
AT4G39260	ATGRP8/GR-RBP8 (COLD, CIRCADIAN RHYTHM, AND RNA BINDING 1, GLYCINE-RICH PROTEIN 8); RNA binding	252885_at	2.00	1.83E-02
DOWNREGULATED GENES				
AT3G30720	unknown protein	256940_at	-9.01	1.12E-11
AT5G48850	male sterility MS5 family protein	248676_at	-4.11	8.29E-04
AT5G01600	ATFER1 (ferretin 1); ferric iron binding	251109_at	-3.57	1.61E-02
AT2G33380	RD20 (RESPONSIVE TO DESSICATION 20); calcium ion binding	255795_at	-3.41	2.52E-03
AT2G37870	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	266098_at	-3.41	1.83E-04
AT4G33550	lipid binding	253344_at	-3.34	4.48E-03
AT3G08860	alanine--glyoxylate aminotransferase, putative / beta-alanine-pyruvate aminotransferase, putative / AGT, putative	258983_at	-3.14	6.18E-04
AT3G55500	ATEXPA16 (*ARABIDOPSIS THALIANA* EXPANSIN A16)	251791_at	-2.85	7.80E-04
AT5G59310	LTP4 (LIPID TRANSFER PROTEIN 4); lipid binding	247718_at	-2.83	1.61E-02
AT3G53980	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	251928_at	-2.82	1.65E-03
AT4G22490	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	254327_at	-2.75	5.59E-04
AT2G32990	glycosyl hydrolase family 9 protein	267595_at	-2.71	2.17E-03
AT3G49580	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT3G49570.1); similar to unknown protein [Brassica rapa subsp. pekinensis] (GB:AAQ92331.1)	252269_at	-2.68	9.51E-03
AT5G24770; AT5G24780	[AT5G24770, VSP2 (VEGETATIVE STORAGE PROTEIN 2); acid phosphatase];[AT5G24780, VSP1 (VEGETATIVE STORAGE PROTEIN 1); acid phosphatase]	245928_s_at	-2.66	2.24E-02
AT4G29700	type I phosphodiesterase/nucleotide pyrophosphatase family protein	253697_at	-2.57	1.32E-02
AT1G64660	ATMGL; catalytic/ methionine gamma-lyase	261957_at	-2.55	3.38E-03
AT1G62540	flavin-containing monooxygenase family protein / FMO family protein	265122_at	-2.53	2.79E-03
AT2G47180	ATGOLS1 (*ARABIDOPSIS THALIANA* GALACTINOL SYNTHASE 1); transferase, transferring hexosyl groups	263320_at	-2.51	5.71E-04
AT3G26960	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT5G41050.1); similar to Os09g0508200 [Oryza sativa (japonica cultivar-group)] (GB:NP_001063620.1); similar to Os12g0472800 [Oryza sativa (japonica cultivar-group)] (GB:NP_001066754.1)	257793_at	-2.42	5.09E-03
AT3G51240	F3H (TRANSPARENT TESTA 6); naringenin 3-dioxygenase	252123_at	-2.42	2.97E-02
AT3G51895	SULTR3;1 (SULFATE TRANSPORTER 1); sulfate transporter	246310_at	-2.26	1.75E-02
AT4G08300	nodulin MtN21 family protein	255127_at	-2.22	2.09E-02
AT1G80760	NIP6;1 (NOD26-like intrinsic protein 6;1); water channel	261881_at	-2.22	1.40E-02
AT3G12580	HSP70 (heat shock protein 70); ATP binding	256245_at	-2.19	4.14E-02
AT5G14760	AO (L-ASPARTATE OXIDASE); L-aspartate oxidase	246597_at	-2.16	1.93E-02
AT5G58390	peroxidase, putative	247812_at	-2.15	1.99E-02
AT2G21560	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT4G39190.1); similar to LOC402866 protein [Danio rerio] (GB:AAH57473.1)	263545_at	-2.15	3.74E-02
AT4G24120	YSL1 (YELLOW STRIPE LIKE 1); oligopeptide transporter	254174_at	-2.13	1.22E-03
AT1G64360	unknown protein	259766_at	-2.12	2.76E-05
AT2G37770	aldo/keto reductase family protein	267168_at	-2.11	2.97E-02
AT2G22240	inositol-3-phosphate synthase isozyme 2 / myo-inositol-1-phosphate synthase 2 / MI-1-P synthase 2 / IPS 2	263433_at	-2.11	3.07E-02
AT1G78970	LUP1 (LUPEOL SYNTHASE 1); lupeol synthase	264100_at	-2.11	3.74E-03
AT3G28270	similar to AT14A [*Arabidopsis thaliana*] (TAIR:AT3G28290.1); similar to AT14A [*Arabidopsis thaliana*] (TAIR:AT3G28300.1); similar to Protein of unknown function DUF677 [Medicago truncatula] (GB:ABE78510.1); contains InterPro domain Protein of unknown functi	256603_at	-2.07	4.01E-02
AT1G17745	PGDH (3-PHOSPHOGLYCERATE DEHYDROGENASE); phosphoglycerate dehydrogenase	259403_at	-2.06	1.99E-02
AT4G28250	ATEXPB3 (*ARABIDOPSIS THALIANA* EXPANSIN B3)	253815_at	-2.05	1.43E-02
AT1G52030; AT1G52040	AT1G52030, MBP2 (MYROSINASE-BINDING PROTEIN 2)];[AT1G52040, MBP1 (MYROSINASE-BINDING PROTEIN 1)	265058_s_at	-2.03	4.18E-02

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PERSONAL DETAILS

Korneel Vandenbroucke

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Place of birth: Roeselare (Belgium)

EDUCATION

Graduated with distinction as master in Biotechnology (2003)

at the University of Ghent, Faculty of Sciences, Department Molecular Genetics

Thesis: Functional analysis of metacaspases in *Arabidopsis thaliana*

Ph.D (2003-2008)

at the University of Ghent, Faculty of Sciences, Department Molecular Genetics

Thesis: Role for hydrogen peroxide during abiotic and biotic stress signaling in plants

SCIENTIFIC PUBLICATIONS

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Oxidative stress regulated gene expression across kingdoms

in Molecular Biology and Evolution 25, 507-516 (2008)

CONTRIBUTIONS TO SCIENTIFIC MEETINGS

Role for H_2O_2 and the oxidative burst during resistance against necrotrophic pathogens

Presented as poster on:

International meeting on Crop Protection (2005);

Ph.D. symposium (2005)

Oxidative stress regulated gene expression across kingdoms

Oral Presentation on:

VIB seminar (2006)

Presented as poster on:

Plant Oxygen Group meeting, Ghent (2007);

Oxygen Meeting, Louvain-la-neuve (2007)

SCIENTIFIC WORKSHOPS

Ensembl workshop, bioinformatics training (2006)

International workshop: increasing tolerance to abiotic stress in plants (Drought stress) (2006)

Technology TransferCourse VIB-UG Department of Plant Systems Biology (2007)

OTHER SCIENTIFIC CONTRIBUTIONS

Supervisor on Scientists@work (2005): What happens in a plant under stress?

Supervisor on practical courses: Genetics and Molecular Techniques, 1ste year master students biology (2006)

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REFERENCES

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Korneel

Nothing shocks me. I'm a scientist.

Harrison Ford as Indiana Jones