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Involvement of a carbohydrate-binding F-box-Nictaba protein from *Arabidopsis thaliana* in plant stress responses

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Image of transgenic *pAt2g02360:GUS Arabidopsis thaliana* plant subjected to GUS assay showing clear staining of the leaf trichomes.

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List of abbreviations

AA	amino acid
ABA	abscisic acid
Ara	arabinose
ASK	Arabidopsis Skp1-like protein
BAP	6-benzylaminopurine
bp	base pairs
BR	brassinosteroid
CaMV	cauliflower mosaic virus
CNX	calnexin
ConA	concanavalin A
CRD	carbohydrate recognition domain
CRT	calreticulin
DNA	deoxyribonucleic acid
dpi	days post infection
EEA	*Euonymus europaeus* agglutinin
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation
ET	ethylene
EUL	*Euonymus* lectin
Fbs	sugar-binding F-box protein
Fuc	fucose
GA	gibberellin
GA_3	gibberellin A3, gibberellic acid
Gal	galactose
GalNAc	*N*-acetylgalactosamine
Glc	glucose
GlcNAc	*N*-acetylglucosamine
GNA	*Galanthus nivalis* agglutinin
GUS	β-glucuronidase
IAA	indole-3-acetic acid
JA	jasmonic acid
kDA	kilodalton
KO	knockout
LacNAc	*N*-acetyllactosamine
LecRK	lectin receptor kinase
LRR	leucine-rich repeat

Man	mannose
MeJA	methyl jasmonate
MS	Murashige and Skoog medium
MW	molecular weight
Nictaba	*Nicotiana tabacum* agglutinin
NLS	nuclear localization signal
OE	overexpression
PBS	phosphate buffered saline
PP2	phloem protein 2
PR	pathogenesis-related
Pst DC3000	*Pseudomonas syringae* pv. *tomato* strain DC3000
pv.	pathovar
qRT-PCR	quantitative reverse-transcriptase polymerase chain reaction
RFU	relative fluorescence units
Rha	rhamnose
RNA	ribonucleic acid
SA	salicylic acid
SCF	Skp1-Cullin1 -F-box protein- Rbx1
Skp1	S-phase kinase-associated protein 1
SD	standard deviation
SE	standard error
SL	strigolactone
T-DNA	transfer DNA
TIR	toll-interleukin-1 receptor
Ub	ubiquitin
UPS	ubiquitin-26S proteasome system
WT	wild type
Xyl	xylose

Scope

Global agriculture faces several challenges; one of the biggest ones is to feed a growing population predicted to reach about 9 billion people by the year 2050. To feed the world population a substantial increase in food production will be required. Unfortunately, the available agricultural land area is not increasing, but is constantly reduced due to industrialization, urbanization or desertification. Furthermore, environmental stresses including drought, extreme temperatures, insect infestation and pathogen infections are major threats to agriculture worldwide leading to tremendous yield losses. While abiotic stresses are usually inherently beyond control, the use of agrochemicals to protect crops from pests and diseases is mostly inefficient and disputable from an environmental point of view. Thus, the demand for more stress-tolerant crops, which would concurrently provide higher yields, is growing. Unfortunately, even though plants have evolved strategies to sense, respond and adapt to adverse growth conditions, our understanding of their genetic and biochemical basis is very limited impeding the use of this natural potential. Nevertheless, the rapid progress in molecular genetic technologies as well as the availability of model plants like *Arabidopsis thaliana* provide invaluable tools for exploring the mechanisms of plant resistance and they are of crucial importance for the ultimate bioengineering of tolerant crops.

In the past decade it became evident that exposure of plants to different environmental stresses results in the synthesis of some specific carbohydrate-binding proteins, called lectins. Recently, a novel family of these plant stress-inducible lectins has been identified, which groups proteins comprising an N-terminal F-box domain and a C-terminal Nictaba lectin domain. The members of this specialized protein family presumably play a crucial role in plant physiology through recognition and regulation of the abundance of key regulatory (glyco)proteins involved in plant responses towards stress. Homologs of the F-box-Nictaba family are widespread in the plant kingdom suggesting a pivotal role.

This study was focused on one of the Nictaba homologs from *Arabidopsis thaliana*, the so-called F-box-Nictaba, which shares the highest sequence similarity with the tobacco lectin Nictaba and thus presumably represents a functional carbohydrate-binding protein. The general aim of this research was to investigate the physiological role of F-box-Nictaba in plant stress responses.

Chapter 1 summarizes the currently available knowledge regarding plant lectins with a special focus on those lectins involved in plant responses to stress. Furthermore, an overview is presented on plant F-box proteins, their role in the molecular control of regulatory proteins and their physiological importance for plants.

The first objective of the work was to characterize F-box-Nictaba at the molecular level. **Chapter 2** describes the recombinant production of the F-box-Nictaba protein and its Nictaba domain in the heterologous expression system *Pichia pastoris* and subsequent protein purification using column

chromatography. The purified proteins have been characterized in some detail, especially with respect to their glycan-binding properties.

The second aim of the research focused on the questions when, where and to what extent the *F-box-Nictaba* gene is expressed in *A. thaliana* plants. **Chapter 3** comprises a detailed expression analysis performed on different plant tissues sampled throughout the development of plants either grown under optimal growth conditions or subjected to various abiotic and biotic stresses. In addition, this chapter includes an *in silico* promoter and co-expression analysis as well as the assessment of *F-box-Nictaba* promoter activity using GUS assays. **Chapter 4** provides information on the expression of *F-box-Nictaba* in specific plant defense-related structures called trichomes.

The third objective of the thesis was to investigate the biological relevance of F-box-Nictaba expression for plant growth and development. Therefore, part of **Chapter 3** deals with the effects of overexpression or reduced expression of *F-box-Nictaba* on plant performance after treatment with specific environmental stresses.

Finally, **Chapter 5** dwells on the findings of this research, their relevance and the implications for our understanding of plant stress responses. This chapter also comprises some perspectives for future studies.

Chapter 1

Introduction

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1.1 Lectins

1.1.1 General introduction

The story of lectins, i.e. the carbohydrate-binding proteins, started at the end of the 19th century when hemagglutinins were first described in castor bean extracts as proteinaceous factors capable of red blood cell agglutination (Stillmark, 1888). Since the first discoveries, these unique proteins have been found widespread in nature ranging from viruses, bacteria and fungi to plants and animals (Esko and Sharon, 2009; Kilpatrick, 2002; Van Damme *et al.,* 2008*;* Varrot *et al.,* 2013). Nowadays, lectins are known as a highly diverse group of proteins of non-immune origin capable of selective recognition and reversible binding to specific carbohydrate structures. Lectins can bind glycans present either in their free form or as a part of glycoconjugates (glycoproteins and glycolipids) without altering the structure of the recognized carbohydrate moiety (Van Damme *et al.,* 2008). Analysis of the amino acid (AA) sequences led to conclusion that the glycan-binding activity of most lectins resides within a confined polypeptide fragment, called the carbohydrate-recognition domain (CRD) (Drickamer, 1988). The ability to agglutinate cells or form precipitates with glycoconjugates, which initially was considered an essential property of lectins, results from the multivalent character of lectins consisting of non-covalently linked glycan-binding subunits and thus containing more than one CRD. It has to be noted, however, that even though the terms agglutinin and hemagglutinin are often used as synonyms, not all lectins are multivalent and thus agglutination is not a requirement to classify a protein as a lectin.

Ever since the beginning of lectin history researchers have been trying to unravel the physiological role of these unique proteins. Throughout the years lectins have been widely exploited to investigate a multitude of biological processes and became a powerful tool in biochemistry and biomedical research (Ghazarian *et al.,* 2011; Fu et al., 2011b; Mislovicova *et al.,* 2009; Peumans and Van Damme, 1998; Rüdiger and Gabius, 2001; Sharon and Lis, 2004). Nevertheless, despite their ubiquity in living organisms and broad scientific application, the biological relevance of many lectins still remains unclear. Due to the high specificity of lectin-carbohydrate interactions, the function of particular lectins is intrinsically linked to the significance of the target glycans in organisms. Glycans and glycoconjugates are known to play a crucial role in many diverse biological events including transcription, translation, protein folding, subcellular localization, intra- and intercellular transport, (in)activation of proteins, cell-molecule interactions, cell-cell communication and defense responses (Varki and Lowe, 2009). Glycosylation is essential for every organism's growth, development or survival and defects in glycan signaling often lead to abnormal development and severe diseases (Boisson *et al.*, 2001; Burn *et al.*, 2002; Gillmor *et al.*, 2002; Ohtsubo and Marth, 2006). Importantly, carbohydrates are no longer regarded solely as an energy reservoir, but are also associated with storage and transfer of biological information. In fact, with respect to the glycan complexity, glycosylation is emerging as a highly complicated multidimensional coding system and the idea of the "sugar code" is recently attracting growing attention (Rüdiger and Gabius, 2009; Pilobello and Mahal,

2007). From this point of view, lectins reveal themselves as specialized proteins which decipher this code, representing the indispensable components of the glycan signaling system assuring that the sweet message is well read and understood.

1.1.2 Glycosylation in plants

Protein glycosylation in plants, as in all eukaryotes, is a major co- and post-translational modification which relies on the covalent linkage of oligosaccharide chain(s) to proteins (Etzler and Mohnen, 2009; Nguema-Ona *et al.,* 2014). Glycosylation mostly occurs on secreted proteins. Depending on the linkage between the glycan moiety and the protein two types of glycosylation are distinguished: *N*- and *O*-glycosylation (Fig. 1.1).

***N*-glycosylation**

N-glycosylation takes place by attachment of the oligosaccharide to the amide nitrogen of an asparagine (Asn) residue within the consensus sequence Asn–X–serine (Ser)/ threonine (Thr) (where X is any amino acid except proline). Most of the proteins which follow the plant secretory pathway contain *N*-linked glycans of four main types: high-mannose, complex, hybrid, and paucimannose (Fig. 1.1B-E) (Etzler and Mohnen, 2009; Nguema-Ona *et al.,* 2014; Strasser, 2014). *N*-glycosylation begins co-translationally in the endoplasmic reticulum (ER) via the transfer of a precursor oligosaccharide $Glc_3Man_9GlcNAc_2$ (Fig. 1.1A) onto specific Asn residues of the nascent polypeptide chain. Then, the *N*-linked glycan is trimmed in the ER by removal of Glc and Man residues to generate high-Man type *N*-glycans ($Man_{5-9}GlcNAc_2$) (Fig. 1.1B). During the glycoprotein passage along the secretory pathway, the high-Man *N*-glycan chain is further processed in the Golgi apparatus by trimming Man residues and adding new sugar residues to generate complex type *N*-glycans (Fig. 1.1C). In some plant glycans, some terminal Man residues are not removed giving rise to the so-called hybrid-type *N*-glycans (Fig. 1.1D). Plant complex *N*-glycan modifications include an α1-3-fucose attached to the proximal *N*-acetylglucosamine (GlcNAc) residue of the core, a β1-2-xylose residue linked to the core β-mannose, a single terminal GlcNAc residue and a terminal-linked fucosylated type-1 lactosamine (LacNAc) (Galβ1-3GlcNAc), called Lewis A (Galβ1-3(Fucα1-4)GlcNAc). Finally, plant glycoproteins processed in the Golgi are either secreted from the cell or are translocated to the vacuoles. Some glycoproteins can also undergo post-Golgi removal of at least the terminal GlcNAc residues giving rise to the paucimannose type *N*-glycans (Fig. 1.1E), which are mostly found on vacuolar glycoproteins.

***O*-glycosylation**

O-glycosylation in plants occurs on the hydroxyl group of hydroxyproline (Hyp), Ser or Thr residues of the polypeptide chain (Nguema-Ona *et al.,* 2014). The most abundant *O*-glycans in plants are found on extensins and arabinogalactan proteins (AGPs) which form the group of Hyp-rich glycoproteins (HRGPs) present in the plasma membrane and in the cell wall (Tan *et al.,* 2012; Velasquez *et al.,* 2012). These proteins are *O*-glycosylated as they pass through the Golgi apparatus via a stepwise

transfer of monosaccharides initiated by the addition of an arabinose (Ara) or galactose (Gal) residue. Extensins are extensively *O*-glycosylated with one to four Ara residues bound to Hyp, and with Gal moieties linked to Ser residues (Fig. 1.1F). AGPs are the most highly glycosylated HRGPs, with Hyp residues frequently glycosylated by branched galactan chains substituted with terminal Ara residues (Fig. 1.1G). Side chains can be further modified with Fuc, rhamnose (Rha), Xyl and other sugars.

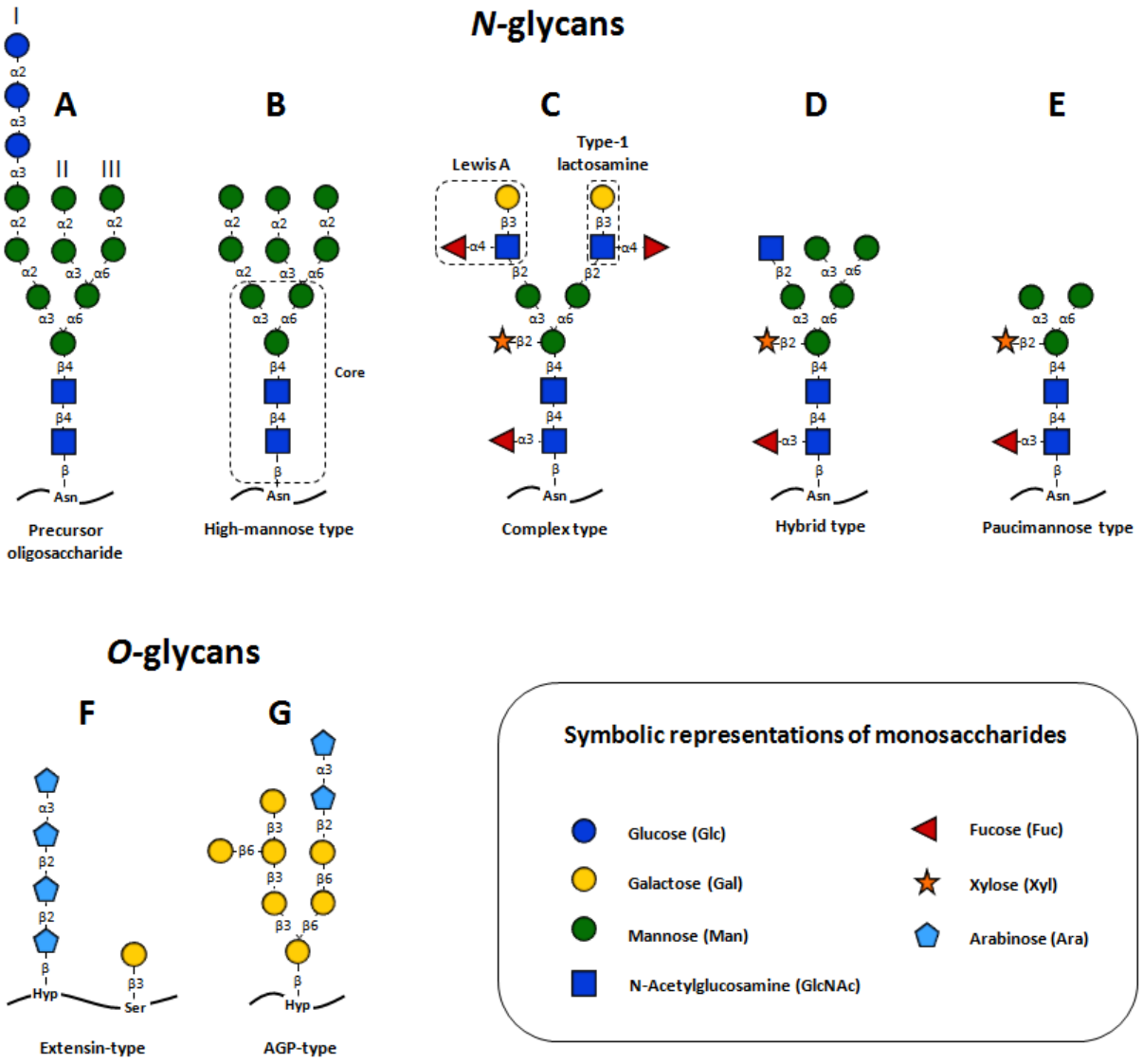


Fig. 1.1 Glycan structures occurring on glycoproteins in plants. A-E, Structures of *N*-glycans. Precursor oligosaccharide $Glc_3Man_9GlcNAc_2$ transferred onto the nascent plypeptide at Asn. Roman numbers I-III indicate different branches of the *N*-glycan (A); High-Man type *N*-glycans composed of the core $Man_3GlcNAc_2$ substituted by two to six Man residues (B); Complex type *N*-glycans present a β1-2-linked Xyl and/or α1-3-linked Fuc bound to the core, or a terminal Galβ1-3(Fucα1-4)GlcNAc trisaccharide (Lewis A) (C); Hybrid type *N*-glycans which contain the GlcNAc residue initiating complex branch I, and Man residues on branches II-III (D); Paucimannose type *N*-glycans (E). F-G, Structures of *O*-glycans. Extensin type *O*-glycans containing one to four Ara residues bound to Hyp, and Gal moieties linked to Ser residues (F); AGP type *O*-glycans containing branched galactan chains substituted with terminal Ara residues linked to Hyp residues (G).

1.1.3 Glycan microarrays

Since carbohydrates and their conjugates are known to have an important function in a variety of biological processes and unraveling of the molecular basis of glycan-protein interactions can provide deep insights into our understanding these glycan-mediated processes, studies of functional glycomics have received a lot of attention in recent years. However, analysis of carbohydrate-protein interactions is difficult due to glycan structure complexity and diversity, as well as low affinity of these interactions. A tool frequently used for exploring carbohydrate-mediated interactions and contributing to significant advances in glycomics is the glycan microarray technology first developed by several research groups in 2002 (Park *et al.,* 2013). Glycan microarrays comprise a wide range of diverse carbohydrates (from natural sources or chemical and enzymatic synthesis) densely and orderly attached to a solid support. Many different types of glycan microarrays have been exploited, in which carbohydrates and glycoconjugates are either covalently or non-covalently immobilized to silica plates, beads or microplates. The arrays differ with respect to the repertoire of glycans included, covering a broad range of different glycan structures (Blixt *et al.,* 2004) or carbohydrate structures representative for the glycome of an organism of interest (e.g. saccharides of the plant cell wall; Pedersen *et al.,* 2012). Their chip-based format allows fast and high-throughput profiling of the glycan-binding properties of carbohydrate-binding molecules (e.g. lectins, antibodies). It is a high-sensitivity platform and requires only minute amounts of samples for quantitative analysis of carbohydrate-protein interactions commonly measured by the detection of fluorophore-labeled proteins (Park *et al.,*2013). As such glycan arrays are a powerful technology widely used in the assessment of carbohydrate-binding properties of antibodies and lectins, in biological and biomedical research for detection of cells and pathogens, identification of anti-glycan antibodies for clinical diagnostics, development of carbohydrate-based vaccines and new drug discovery (Katrlík *et al.,* 2011; Liang and Wu, 2009; Park *et al.,* 2008).

1.1.4 Plant lectins

Throughout over 100 years of research on carbohydrate-binding proteins, more than 500 highly diverse lectins from many different plants (including important crops like tomato, wheat, potato, rice, bean and soybean) have been isolated and characterized (Van Damme *et al.,* 1998). With respect to the differences in their molecular structure, glycan-binding specificity, biological activity as well as regulation, plant lectins constitute an extremely heterogenous group and for years this diversity was problematic for the appropriate and functional classification of these proteins. Nonetheless, structural and molecular studies allowed to identify a limited number of different CRDs in plants which were used as basic structural units to group structurally and evolutionarily related lectins. Based on this criterion, plant lectins have been clustered into 12 families (Van Damme *et al.,* 2008) as presented in Table 1.1.

Table 1.1 Overview of the 12 plant lectin families grouped based on the structural and evolutionary related glycan-binding domains. Families comprising nucleocytoplasmic lectins are marked in gray.

Lectin family	Taxonomic distribution		Structure	Specificity(ies) within the family	Example(s)
	Vacuolar lectins	Nucleocytoplasmic lectins			
***Agaricus bisporus* agglutinin orthologs**	No examples known	Documented in *Marchantia polymorpha* and *Tortula ruralis*	Homodimer β-sandwich	T-antigen (Galβ1-3GalNAc) GlcNAc	**MarpoABA** (*Marchantia polymorpha*)
Amaranthins	No examples known	Documented in *Amaranthus* spp and *Prunus* spp	Homodimer β-trefoil	T-antigen and GalNAc	**Amaranthin** (*Amranthus caudatus*)
Class V chitinase homologs	Documented in a few legumes	No examples known	Homodimer TIM-barrel	High-Man *N*-glycans	**RobpsCRA** (*Robinia pseudoacacia*)
Cyanovirins	Documented in *Selaginella moellendorffii, Ceratopteris richardii and Marchantia polymorpha*	No examples known	Homodimer Triple-stranded β-sheet and β-hairpin	High-Man *N*-glycans	**CrCVNH** (*Ceratopteris richardia*)
EUL-related proteins	No examples known	Ubiquitous	Homodimer β-trefoil (predicted)	Blood group B motifs (Galα1–3(Fucα1–2)Galβ1–4GlcNAc) and high-Man *N*-glycans	**EEA** (*Euonymus europaeus*)
				Lewis X (Galβ1-4(Fucα1-3)GlcNAc), Lewis Y (Fucα1-2Galβ1-4(Fucα1-3)GlcNAc), lactosamine (Galβ1-4GlcNAc)	**ArathEULS3** (*Arabidopsis thaliana)*
GNA-related lectins	Wide taxonomic distribution	Found in diverse taxa	Different oligomerisation states β-barrel	oligoMan and high-Man *N*-glycans	**GNA** (*Galanthus nivalis*)
				Complex *N*-glycans	**GNA_{maize}** (*Zea mays*)
Jacalin-related lectins	Documented in a few Moraceae spp	Ubiquitous	Different oligomerisation states β-prism	Gal/T-antigen	**Jacalin** (*Artocarpus heterophyllus*)
				high-Man and complex *N*-glycans	**Orysata** (*Oryza sativa*)
Lectins with hevein domains	Wide taxonomic distribution	No examples known	Different oligomerisation states Hevein domain	$(GlcNAc)_n$, Sia	**WGA** (*Triticum vulgaris*)
LysM-related proteins	Wide taxonomic distribution	No examples known	Different oligomerisation states β-α-α-β structure	$(GlcNAc)_n$	**CERK1** (*Arabidopsis thaliana*)
Nictaba-related lectins	No examples known	Wide taxonomic distribution	Homodimer β-sandwich (predicted)	$(GlcNAc)_n$, high-Man and complex *N*-glycans	**Nictaba** (*Nicotiana tabacum*)
Proteins with legume lectin domains	Wide taxonomic distribution, common in Fabaceae and Lamiaceae	No examples known	Different oligomerisation states β-sandwich	Man/Glc, Gal/GalNAc, $(GlcNAc)_n$, Fuc, Siaα2,3 Gal/GalNAc, complex glycans	**ConA** (*Canavalia ensiformis*)
Ricin-B lectins	Wide taxonomic distribution	No examples known	Different oligomerisation states β-trefoil	Gal/GalNAc	**Ricin** (*Ricinus communis*)

Fuc, fusose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Man, mannose; Sia, sialic acid;

Due to practical constraints, for years lectinology was mainly focused on the lectins which could be detected easily in plants by agglutination assays – these are lectins constantly present in reasonably high quantities in specific plant tissues and mainly localized in the vacuole of plant cells or in the intercellular compartment. Nevertheless, studies of recent years have brought evidence that some plant lectins are occurring in the nucleus and the cytoplasm of plant cells and are not permanently produced, but are expressed only under particular conditions. Even then, their content in plants remains rather low. These observations founded the concept of two functionally distinct groups of plant lectins: the classical vacuolar lectins and the inducible nucleocytoplasmic lectins (Lannoo and Van Damme, 2010; Van Damme *et al.,* 2004). However, there is also a group of chimeric lectin-like proteins which are neither vacuolar nor nucleocytoplasmic but are mainly localized in the plasma membrane (Vaid *et al.,* 2013).

1.1.4.1 Vacuolar lectins

Most classical lectins are constitutively produced in the plant cell at relatively high concentrations (0.1 – 10% of the total protein amount) and their presence is independent from environmental conditions. They are synthesized with a signal peptide on endoplasmic reticulum (ER)-associated ribosomes and as such they are transported via the Golgi apparatus either to the vacuole or, following the secretory pathway, accumulate in the extracellular compartment. These lectins are principally found in seeds as well as in vegetative storage tissues of plants like tubers, bulbs and rhizomes (Van Damme *et al.,* 2008). High levels of lectins are usually found in legumes (i.e. beans including peanuts). The majority of the vacuolar lectins exhibit carbohydrate-binding specificity towards glycans mainly occurring outside plants (i.e. complex animal *N*- and *O*-glycans) (Peumans *et al.*, 2000a).

1.1.4.2 Nucleocytoplasmic lectins

Studies of the last years have unambiguously demonstrated that apart from the vacuolar lectins abundantly synthesized in specific (storage) tissues, plants also produce minute amounts of lectins which are exclusively found in the nucleus and the cytoplasm of non-storage organs (leaves, roots, flowers) (Lannoo and Van Damme, 2010; Van Damme *et al.,* 2004). Furthermore, these so-called nucleocytoplasmic lectins are not constantly expressed, but are synthesized in very low quantities only upon plant exposure to specific stress conditions such as drought, salt, wounding, treatment with plant hormones, pathogenic infection or insect herbivory. Since these lectins are not detectable in plant tissues during normal growth conditions but are clearly up-regulated after application of physical, chemical or biotic stress factors, this lectin group is referred to as the inducible plant lectins (Van Damme *et al.,* 2011). Initially, the concept of nucleocytoplasmic lectins was mainly based on the data from rice and tobacco, where the first inducible lectins, in particular *Oryza sativa* agglutinin (Orysata) and *Nicotiana tabacum* agglutinin (Nictaba), were discovered (Zhang *et al.,* 2000; Chen *et al.,* 2002). At present however, it becomes obvious that at least some nucleocytoplasmic lectins are widespread throughout the plant kingdom (Lannoo and Van Damme, 2010). Until now

nucleocytoplasmic carbohydrate-binding proteins have been identified within 6 out of the 12 plant lectin families listed in Table 1.1 (marked in grey), namely in the family of *Agaricus bisporus* agglutinin orthologs, amaranthins as well as the *Euonymus europaeus* lectin- (EUL-), *Galanthus nivalis* agglutinin- (GNA-), jacalin- and Nictaba-related lectins. Each of these lectin families are briefly presented below, but since this PhD research is mainly focused on a homolog belonging to the family of Nictaba-related carbohydrate-binding proteins, two separate sections are especially dedicated to discuss the prototype lectin Nictaba (1.1.3) and its homologs (1.1.4) in more detail.

***Agaricus bisporus* lectin orthologs**

The prototype of this family is actually a typical fungal lectin, the *Agaricus bisporus* lectin from the edible mushroom *Agaricus bisporus*. The protein comprises two glycan-binding sites with distinct specificity for T-antigen (Galβ1-3GalNAc) and GlcNAc-exposed N-linked glycans (Nakamura-Tsuruta *et al.,* 2006). Plant homologs of *Agaricus bisporus* lectin are restrained to lower plants and until now their expressed sequences have only been reported in the liverwort *Marchantia polymorpha* and in the moss *Tortula ruralis.* Homologs from *M. polymorpha* (called MarpoABA) have been demonstrated to present comparable lectin specificity as the fungal prototype (Peumans *et al.,* 2007). Moreover, localization studies showed that MarpoABA is present in the cytosol and in the nucleus of plant cells.

Amaranthins

Amaranthins constitute a small family called after the first lectin discovered in *Amaranthus caudatus* seeds (Rinderle *et al.*, 1989). Homologs have been found in different *Amaranthus* species but amaranthin-like sequences have also been reported outside the Amaranthaceae family (Van Damme *et al.,* 2008). It has been shown that amaranthin exhibits glycan-binding specificity towards T-antigen (Galβ1-3GalNAc) (Transue *et al.,* 1997). Based on the AA sequence which lacks a signal peptide and on localization experiments, it has been concluded that amaranthin-like lectins are most probably nucleocytoplasmic proteins (Van Damme *et al.,* 2009).

EUL-related proteins

The *Euonymus europaeus* agglutinin (EEA), which was originally found in the arils of spindle tree (Petryniak *et al.,* 1977), has been recognized as the first representative of a new family of proteins containing (an) EUL domain(s) (Fouquaert *et al.*, 2008). EEA has been shown to exhibit a dual glycan-binding specificity towards blood group B oligosaccharides as well as (with lower affinity) high-mannose(Man) *N*-glycans. Localization studies have demonstrated its presence in the nucleus and cytoplasm of plant cells (Van Hove *et al*., 2011). Screening of plant genome databases revealed that the EUL domain equivalent to the EEA polypeptide is ubiquitous in Embryophyta ranging from lower plants like mosses, to monocots and dicots (Fouquaert *et al.*, 2009a; Fouquaert and Van Damme, 2012). Some of the identified putative EUL-related proteins consist of a single EUL domain only or of two tandem-arranged EUL domains, while others comprise also an additional unrelated N-terminal sequence. The EUL-like proteins studied until now differ in their glycan-binding specificity from EEA (Fouquaert and Van Damme, 2012). Depending on the homolog, they preferentially recognize

galactosylated structures, as shown for OrysaEULD1A (Al Atalah *et al.,* 2014c) and ArathEULS3 (Van Hove *et al.,* 2011), or high-Man *N*-glycans e.g. OrysaEULS2 (Al Atalah *et al.,* 2012). Localization assays with enhanced green fluorescence protein (EGFP) fusion proteins revealed that, similar to the EEA, EUL-related lectins from *Arabidopsis thaliana* and rice are located in the cytoplasm and also in the nucleus of plant cells (Al Atalah *et al.,* 2013; Van Hove *et al.,* 2011).

GNA-related lectins

The *Galanthus nivalis* agglutinin (GNA), a Man-specific lectin, was originally isolated from the bulbs of *Galanthus nivalis* (snowdrop) (Van Damme *et al.,* 1987). While it reacts weakly with Man, it exhibits strong affinity towards oligomannosides and high-Man *N*-glycans (Van Damme *et al.,* 2008). Most plant GNA-related proteins are synthesized with an N-terminal signal peptide (Van Damme *et al.,* 2007). Hence, as it has been reported for GNA (Fouquaert *et al.,* 2007), they are most probably targeted to the vacuole. Nevertheless, genomic analyses revealed the presence of GNA-related proteins in maize, wheat, rice and *Medicago truncatula* which are not flanked with an N-terminal signal peptide and as such are considered to be putative nucleocytoplasmic GNA-like proteins (Van Damme *et al.,* 2004). Localization studies confirmed that GNA homologs from maize and rice locate to the nucleocytoplasmic compartment of the plant cell (Fouquaert *et al.,* 2007). Furthermore, as demonstrated by analyses on a GNA homolog from maize which recognizes complex *N*-glycans instead of high-Man *N*-glycans, the binding properties of GNA-related lectins can differ significantly (Fouquaert *et al.,* 2009b).

Jacalin-related lectins

The family of the jacalin-related lectins is called after jacalin, a T-antigen-specific lectin isolated from the seeds of jack fruit (*Artocarpus integrifolia*) (Sastry *et al.,* 1986). It groups proteins with one or more domains homologous to jacalin and forms a very heterogeneous family as the members differ substantially in their molecular structure as well as in their carbohydrate-binding specificity. Based on the disparity within the family, it is divided into two groups: (1) galactose (Gal)-specific lectins identified exclusively in Moraceae and (2) Man-specific lectins widespread in the plant kingdom (Van Damme *et al.,* 2008). While Gal-binding jacalin-related proteins are targeted into the vacuolar compartment (e.g. jacalin itself), Man-binding jacalins are synthesized without a signal peptide on free ribosomes and as such accumulate in the cytoplasm. Examples of the latter group are Calsepa, a jacalin-related protein from *Calystegia sepium* (Peumans *et al.,* 2000b) and Orysata, a Man-binding jacalin homolog from rice (Zhang *et al.,* 2000).

1.1.4.3 Chimeric proteins with a lectin domain

Screening of publicly available plant genomes and transcriptomes has demonstrated that, except for the *A. bisporus* lectin domain, all nucleocytoplasmic lectin domains identified until now occur as part of chimeric protein sequences. In other words, apart from the glycan-binding domain, these so-called

chimerolectins comprise also other unrelated domain(s) (Lannoo and Van Damme, 2010; Van Damme *et al.,* 2008).

Within the amaranthin family only a few putative chimerolectins have been found. In *Zea mays* and *Triticum aestivum* proteins comprising amaranthin domain(s) C-terminally linked to an aerolysin-like domain have been identified. Also, an amaranthin-related protein with a C-terminal kinase-like domain has been found in *Aquilegia formosa x Aquilegia pubescens*. Although most GNA homologs in plants are built up of GNA domains exclusively, a couple of putative nucleocytoplasmic chimerolectins have been identified with a GNA-like domain (Van Damme *et al.,* 2008). In contrast, within the family of EUL-related lectins many sequences comprise an extra unrelated and highly variable in size N-terminal domain, and some EUL domain proteins are also characterized by an unrelated C-terminal domain. Likewise, jacalin-related chimerolectins with additional N- or C-terminal domain(s) turn out to be widespread in plants. These extra unrelated domains include different N-terminal motifs like 'dirigent' domains in grass species (Jiang *et al.,* 2007; Kittur *et al.,* 2007; Kittur *et al.,* 2009; Yong *et al.,* 2003), F-box domains in *A. thaliana* (Nagano *et al.*, 2008), a kinase domain in *O. sativa* or a combination of an NB-ARC with a leucine-rich repeat (LRR) domain in *O. sativa*. Moreover, a jacalin-like chimerolectin with C-terminal tandem-arranged Kelch motifs has been found in *A. thaliana* (Van Damme *et al.,* 2008).

For most of the identified putative nucleocytoplasmic chimerolectins the carbohydrate-binding activity of the lectin domain is merely hypothetical, because the proteins have not yet been purified and characterized. The exceptions include e.g. ArathEULS3 (Van Hove *et al.,* 2011), OrysaEULD1A (Al Atalah *et al.,* 2014c), horcolin (Grunwald *et al.,* 2007), VER2 (Xing *et al.,* 2009), Calsepa and MornigaM (Nakamura-Tsuruta *et al.,* 2008), which have been demonstrated to be functional lectins. Furthermore, only a couple of them are experimentally confirmed nucleocytoplasmic lectins (Lannoo, 2007; Van Hove *et al.,* 2011) and despite the absence of a signal peptide, for most chimeroproteins localization in the cytoplasm and nucleus of plant cells still needs to be proven. As a consequence, the physiological role(s) of those lectin-related chimeric proteins is an open question.

1.1.4.4 Membrane-associated proteins containing a lectin-like domain

A unique group of plant chimeric lectins are receptor-like kinases (RLKs) containing a putative glycan-binding domain. They are referred to as lectin receptor kinases (LecRKs) and are involved in plant defense signaling (for details regarding their physiological relevance see section 1.1.2.5). Typically, LecRKs are localized in the plasma membrane and consist of a highly variable N-terminal extracellular carbohydrate-binding-like domain, a conserved C-terminal intracellular (cytosolic) Ser/Thr kinase domain and a hydrophobic membrane-spanning motif in between. LecRKs form a large and extremely heterogeneous family containing diverse types of putative lectin motifs (Barre *et al,* 2002; Vaid *et al.,* 2012). They are classified into 4 types with respect to their lectin domain referred to as C-, G-, L-, and LysM-type LecRKs (Singh and Zimmerli, 2013; Vaid *et al.*, 2013).

C-type (calcium-dependent) LecRKs are scarce in plants and so far as little as only one member has been identified in *A. thaliana*, though its function has not been elucidated yet (Bouwmeester and

Govers, 2009). In contrast, based on genome/transcriptome data, G-type LecRKs with an extracellular GNA-like domain are ubiquitous among Embryophyta (Van Damme *et al.,* 2008). In Arabidopsis and in rice 32 and 100 representatives have been identified, respectively (Vaid *et al.,* 2012). G-type LecRKs are also known as S-locus RLKs since they contain an S-domain known for its involvement in self-incompatibility (SI) in flowering plants (Sherman-Broyles *et al.,* 2007). Representatives of the L-type LecRKs containing a legume-like lectin domain have been described in *A. thaliana* (45 members; Bouwmeester and Govers, 2009), in tobacco (Kanzaki *et al.*, 2008; Gilardoni *et al.*, 2011), and *M. truncatula* (Navarro-Gochicoa *et al.*, 2003). The Arabidopsis L-type LecRKs are characterized by a differential expression in tissues, throughout developmental stages as well as upon stress treatment (Bouwmeester and Govers, 2009). Finally, LysM-type LecRKs with extracellular LysM domains, are found in many plants including leguminous plants, Arabidopsis, rice and poplar (Zhang *et al.,* 2007). The LysM domain is a ubiquitous protein motif first identified in microbial hydrolases which are involved in bacterial cell wall degradation. Nowadays, the LysM motif is considered to bind various types of bacterial peptidoglycans and fungal chitin through recognition of the GlcNAc moieties (Buist *et al.*, 2008; Gust *et al.*, 2012).

1.1.4.5 Physiological roles of plant lectins

One of the major issues regarding lectins is their physiological role in plants. Despite the vast distribution of lectins in the plant kingdom, the biological relevance of most of these carbohydrate-binding proteins remains an open question. Nonetheless, during the last decade, substantial progress has been made following the discovery of inducible lectins localized in the nucleus and the cytoplasm of plants cells. The concept of nucleocytoplasmic glycan-binding proteins put the research on the physiological function(s) of plant lectins in a totally different perspective. The hitherto prevalent image of lectins being merely storage/defense proteins has suddenly developed into the idea whereby lectins are considered as highly specialized molecules involved in complex signaling processes within the plant cell (Lannoo and Van Damme, 2014; Van Damme *et al.,* 2004; Van Damme *et al,* 2011). Overview of physiological roles of plant lectins is presented in Fig. 1.2.

Storage/defense function

Since the classical (vacuolar) lectins are abundantly present in plant storage tissues including seeds as well as vegetative organs like tubers, bulbs and rhizomes, and are constitutively produced in the plant cell in relatively high amounts they are commonly accepted to fulfill a role as storage proteins (Van Damme *et al.,* 2008). Nevertheless, the majority of them recognize complex animal *N*- and *O*-glycans rather than plant-type glycans (Peumans *et al.*, 2000a). As such, they could potentially bind glycans from other (harmful) organisms and consequently play a role in plant defense. In fact, feeding assays and experiments with transgenic plants over-expressing specific lectins have demonstrated that some of these lectins (e.g. concanavalin A (ConA), GNA, wheat germ agglutinin WGA)) exhibit adverse effects on pest insects belonging to the orders Lepidoptera, Coleoptera, Diptera and Hemiptera by negatively affecting their development and survival (Michiels *et al.,* 2010;

Vandenborre *et al.,* 2011), and as a result these lectins enhance plant resistance. Therefore, apart from the endogenous storage function, it is hypothesized that they might have an alternative role in plant defense reactions in the circumstances of predator attack (Van Damme, 2008).

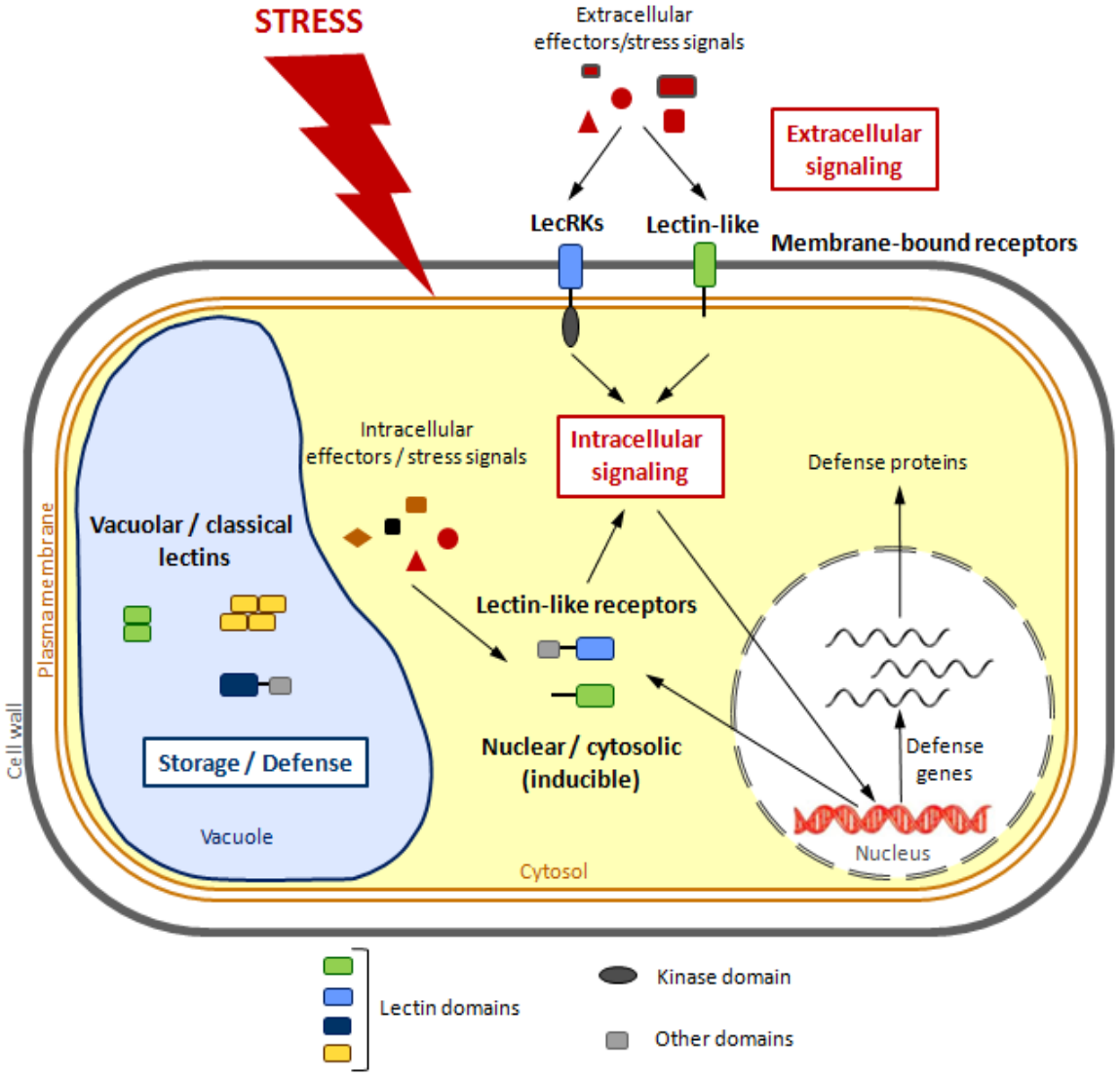


Fig. 1.2 Overview of physiological roles of different types of lectins in plant cells. The classical plant lectins localized in the vacuoles of plant cells play a storage and/or defense role. Membrane-bound LecRKs and lectin-like receptors function in stress signaling by recognizing extracellular effectors generated during stress challenge. After recognition, the signal is transmitted from the outside to the inside of the cell via cytosolic (kinase) domains of membrane-bound lectin-related receptors which activate the downstream intracellular signaling pathways. The inducible lectin-like receptors localized in the nucleus and/or cytosol of plant cells play a role in intracellular stress signaling through recognition of intracellular stress signals. Activation of the downstream intracellular signaling pathways mediated by lectin-like receptors leads to transcription of defense gene and synthesis of defense proteins, which ultimately results in plant defense response against adverse extracellular conditions.

Role in plant stress signaling

Plants are constantly challenged with a multitude of environmental stresses both of abiotic (heat/cold, drought, salt) as well as biotic (pathogen and insect attacks) origin (Ahuja *et al.,* 2010; Atkinson and Urwin, 2012; Rasmussen *et al.,* 2013). Since they are sessile organisms and cannot avoid adverse conditions, plants were forced to develop a wide assortment of sophisticated and tightly regulated mechanisms allowing them rapid adaptation to a changing environment (Golldack *et al.,* 2014; Muthamilarasan and Prasad, 2013; Osakabe *et al.,* 2013; Qu *et al.,* 2013; Wirthmueller *et al.,* 2013). Precise and more importantly prompt perception and transduction of external signals resulting in an effective response to particular stimuli is essential for plant survival under unfavorable circumstances. As a consequence, fine-tuned plant defense responses to environmental challenges rely on a plethora of specific molecules including proteins which play a role in stress signaling. Among these proteins lectins turn out to be of particular importance including the LecRKs localized in the plasma membrane, responsible for perceiving the specific extracellular signal and transmitting it into the cell, as well as the stress-responsive nucleocytoplasmic lectins (Lannoo and Van Damme, 2014) which could further transduce the information inside the cell or affect the transcription/activity of other defense-related genes/proteins, ultimately leading to an enhanced plant resistance to specific stress conditions.

Extracellular signal perception and transduction

Even though plant transmembrane LecRKs form a large protein family, so far only a small number is functionally characterized and it remains unclear whether the lectin domain is functional and if its glycan-binding activity is relevant for the physiological role of the receptor. Nonetheless, it is hypothesized that LecRKs play a role in diverse signaling processes (particularly in immunity) as pattern recognition receptors (PRRs) by selective perception and binding of specific stress-related glycan motifs present extracellularly (Lannoo and Van Damme, 2014; Macho and Zipfel*,* 2014). In case of e.g. pathogenic infection these unique carbohydrates may originate either from the attacking pathogen (then these glycans are called pathogen- or microbe-associated molecular patterns - PAMPs/MAMPs) or from the damaged cell wall structures of the plant itself (the so-called damage-associated molecular patterns - DAMPs) (Newman *et al.*, 2013; Nühse, 2012; Wirthmueller *et al.*, 2013). After recognition of such specific glycan molecules, LecRKs could further transmit the signal from the outside to the inside of the cell via their cytoplasmic kinase domain by activating downstream intracellular signaling pathways (including activation of the mitogen-activated protein kinases (MAPK) and reactive oxygen species (ROS) production) which eventually should result in an appropriate stress-dependent defense response of the cell (Muthamilarasan and Prasad, 2013; Osakabe *et al.,* 2013).

Indeed, recent studies have brought evidence that some of the LecRKs are involved in plant defense responses against (a)biotic stimuli. Until now G-type LecRKs, well known for their involvement in SI reactions in flowering plants (Sherman-Broyles *et al.,* 2007), have additionally been shown to play a role in plant stress responses to abiotic (Sun *et al.*, 2013) and biotic stress (Chen *et al.,* 2006; Kim *et al.*, 2010a). In Arabidopsis, L-type LecRKs are involved in plant defense against bacterial and fungal

pathogens (Bouwmeester *et al.,* 2011; Huang *et al.*, 2013, 2014; Singh *et al.*, 2012), abscisic acid (ABA) signaling and stomatal regulation (Paparella *et al.*, 2014; Singh *et al.*, 2012), as well as in salt and osmotic stress (Dang *et al.,* 2009). In tobacco, L-type LecRKs have been shown to play a role in plant resistance to pathogens and insects (Kanzaki *et al.*, 2008; Gilardoni *et al.*, 2011). Furthermore, the LysM-type chitin elicitor receptor kinase 1 from Arabidopsis (AtCERK1) and its ortholog from rice (OsCEBiP) turn out to be the major chitin-sensing receptors crucial for plant antifungal responses (Brotman *et al.,* 2012; Kouzai *et al.*, 2014; Petutschnig *et al.*, 2010; Shinya *et al.*, 2012; Tanaka *et al.*, 2013). In fact, LecRKs are relevant not only for plant defense responses against harmful organisms, but also play an important role in symbiotic interactions between leguminous plants and beneficial bacteria and fungi. Mostly LysM but also L-type LecRKs have been shown to perceive mycorrhizal fungi and rhizobacteria by recognizing rhizobial lipochitin-oligosaccharide signals or Nod factors involved in nodulation and rhizobial symbiosis (Gust *et al.*, 2012; Knogge and Scheel, 2006, Limpens et al., 2003; Navarro-Gochicoa *et al.,* 2003; Radutoiu *et al.,* 2003).

Lastly, apart from the role in plant interactions with foreign organisms, LecRKs may be relevant for developmental processes. Wan *et al.* (2008) have demonstrated that the L-type LecRK called SGC lectin is required for proper pollen development in Arabidopsis. Other LecRKs are involved in regulation of the ABA response during seed germination (Deng *et al.*, 2009; Xin *et al.*, 2009). Moreover, very recently, the first identified plant ATP receptor DORN1 required for ATP-mediated responses in Arabidopsis was shown to be an L-type LecRK (Choi *et al*., 2014). This same protein was already known as a mediator of cell wall-plasma membrane integrity, yet the latter function relies on protein-protein interactions (Gouget *et al.*, 2006).

Intracellular signaling in the nucleus and cytoplasm

As described in previous sections, plants synthesize specific inducible lectins which are localized in the nucleus and cytoplasm of plant cells at very low concentrations (Lannoo and Van Damme, 2010; Van Damme *et al.,* 2011). These nucleocytoplasmic lectins are expressed in plants under particular stress challenges such as drought, salt, wounding, application of plant hormones, microbial infection or insect herbivory. With respect to their nucleocytoplasmic localization, stress-inducible synthesis and low, but still physiologically relevant concentration, it is hypothesized that they function in the plant cell as components of the intracellular stress signaling pathways, presumably by recognizing and binding specific glycan structures. Obviously, to get a better insight into the role of these lectins in plants, it is crucial to identify possible lectin ligands within plant cells. Yet, knowledge regarding the glycans and glycoconjugates present in the nucleus and cytoplasm is rather scarce (Funakoshi and Suzuki, 2009) and possible endogenous functions of nucleocytoplasmic lectins still remain mostly speculative. Nevertheless, some of these lectins have already been partially characterized in view of their carbohydrate-binding activity and possible physiological role in plant.

Within the amaranthin family a wheat-specific lectin containing a C-terminal aerolysin motif displays up-regulation upon insect feeding (Puthoff *et al.,* 2005). Interestingly, over-expression of amaranthin in different plant species increases plant's resistance to aphids by affecting growth and development of insects (Wu *et al.*, 2006; Xin *et al.*, 2011).

Over the last decade, evidence was accumulating that the expression of EUL proteins is triggered by specific stress factors including ABA application, drought, salt and osmotic challenges (Carpentier *et al.,* 2007; Dooki *et al.,* 2006; Fouquaert *et al.*, 2009a; Kawasaki *et al.,* 2001; Moons *et al.,* 1997a; Riccardi *et al.,* 2004). Very recently Van Hove *et al.* (2014) have shown that an EUL-related protein from *A. thaliana* (ArathEULS3) is significantly up-regulated after ABA application and salt stress. It has also been demonstrated that ArathEULS3 interacts with the nuclear/cytosolic ABA receptor RCAR1 as well as with a calcium-dependent kinase CPK3 involved in the ABA response in guard cells (Li *et al*., 2014a; Berendzen *et al.*, 2012), pointing towards the probable role of ArathEULS3 in ABA signaling and stomatal movements. Moreover, several rice EUL-related lectins (OrysaEULD1A, OrysaEULD1B and OrysaEULD2A) are inducible upon ABA and salt stress, and could be involved in plant response towards biotic stress (Al Atalah *et al.,* 2013; Al Atalah *et al.,* 2014a). Although the exact physiological function of nucleocytoplasmic EUL lectins still remains unclear, their wide distribution in the plant kingdom as well as the stress-dependent expression of EUL-related proteins both strongly suggest that the EUL domain probably plays a general role in stress-associated biological processes in the nucleus and the cytoplasm of plant cells.

Also jacalin-related proteins are playing a role in plant stress responses since numerous members of this family are responsive to a multitude of environmental stimuli. The rice lectin Orysata is up-regulated by salt, drought stress, jasmonic acid (JA) and ABA application as well as after bacterial infection (Al Atalah *et al.,* 2014a; de Souza Filho *et al.,* 2003; Qin *et al.,* 2003; Moons *et al.,* 1997b). What is more, expression of Orysata has been demonstrated to enhance plant resistance to fungal infection and insect herbivory (Al Atalah *et al.*, 2014b; Shinjo *et al.,* 2011). Some other jacalin-like lectins are also involved in plant defense responses against fungi, viruses and herbivores (Nagano *et al.*, 2008; Regente *et al.,* 2014; Xiang *et al.*, 2011; Yamaji *et al.*, 2012). Chimeric jacalins containing a dirigent domain found in Poaceae family are induced upon vernalization, JA and ABA treatment and insect herbivory (Feng *et al.*, 2009; Ma *et al.*, 2013; Song *et al.,* 2014; Williams *et al.,* 2002; Yong *et al.,* 2003). Importantly, some of them increase plant resistance to bacterial, fungal, and viral pathogens as well as to insects (Chisholm *et al.,* 2001; Ma *et al.*, 2010; Subramanyam *et al.*, 2008). A rice jacalin-related protein with a dirigent domain, OsJAC1, is important for rice growth and development (Jiang *et al.*, 2007). Another jacalin-related lectin VER2 plays a role in sensing prolonged cold in wheat (Xiao *et al.,* 2014).

Finally, also the nucleocytoplasmic tobacco lectin Nictaba (described in more detail together with Nictaba-like proteins in sections 1.1.3-1.1.4) is inducible upon specific stress treatments including jasmonate application and insect herbivory (Chen *et al.,* 2002; Lannoo *et al.,* 2007; Vandenborre *et al.*, 2009a, 2009b). Furthermore, it has been demonstrated to exhibit entomotoxic activity (Vandenborre *et al.*, 2010, 2011). Remarkably, Nictaba interacts with histone proteins. Therefore it is suggested that Nictaba could regulate expression of defense-associated genes through binding to chromatin via O-GlcNAcylated histone proteins (Delporte *et al.,* 2014a; Schouppe *et al.,* 2011). Unfortunately, information regarding the physiological relevance of the other putatively nucleocytoplasmic proteins belonging to the Nictaba family is very limited (Delporte *et al.,* 2015).

Protein quality control

Newly synthesized proteins as well as those already present in cells are constantly at risk of misfolding and aggregation. Since the accumulation of abnormal proteins in plants can lead to perturbations in cell homeostasis, pathological states, and even cell death (Deng *et al.,* 2013; Eichmann and Schafer, 2012; Howell, 2013), eukaryotic cells have developed a sophisticated ER protein quality control mechanism for protection from such danger (Liu and Li, 2014). The process takes place predominantly in the ER and relies on two glucose(Glc)-binding lectins acting as molecular chaperones: calnexin (CNX) and calreticulin (CRT). Upon entering the ER, nascent polypeptides of secretory and membrane proteins are co-translationally *N*-glycosylated with the $Glc_3Man_9GlcNAc_2$ motif. Almost immediately after glycosylation, the two terminal Glc residues are cleaved off by glucosidases leaving a $GlcMan_9GlcNAc_2$ – a carbohydrate structure specifically recognized by a membrane-bound CNX and its ER luminal homolog CRT. CNX and CRT act together to monitor and assist in proper folding of the protein. As soon as the folding process is successfully completed, the last Glc residue is removed from the oligosaccharide and the correctly folded protein is released for further processing. However, in case of folding failure, a typical *N*-glycan chain on misfolded or incompletely assembled proteins gets trimmed by mannosidases (Hüttner *et al.,* 2014). Such proteins ultimately modified with $Man_{3\text{-}9}GlcNAc_2$ structures are targets for the ER-associated protein degradation (ERAD) pathway and get eliminated from the cell via the ubiquitin (Ub)–proteasome system (UPS) (Hüttner and Strasser, 2012; Strasser, 2014). The ER is a highly controlled and stress-susceptible cellular compartment and thus under adverse environmental conditions, when misfolded proteins are accumulating, the so-called unfolded protein response is activated, which induces the synthesis of factors promoting folding or removing incorrectly folded proteins through ERAD (Deng *et al.,* 2013; Eichmann and Schafer, 2012; Howell, 2013).

Glycoprotein degradation

Following the ERAD pathway, misfolded glycoproteins carrying $Man_{3\text{-}9}GlcNAc_2$ structures get translocated from the ER into the cytosol, where they are specifically recognized by a sugar-binding F-box (Fbs) protein and consequently get labeled with Ub for proteasomal degradation. The Fbs proteins have been well described in mammals (Yoshida, 2007), but evidence for their existence and functionality in plants is still missing. Nonetheless, it has been shown that plants do express F-box proteins with lectin-like domains (*in casu* jacalin or Nictaba domains) which presumably play a similar role in plants (Lannoo *et al.,* 2008). These putative carbohydrate-binding F-box proteins are discussed in more detail in section 1.2.4.

1.1.5 Nictaba – a lectin from tobacco

1.1.5.1 Inducible expression of Nictaba

Nicotiana tabacum agglutinin, abbreviated as Nictaba, has been discovered in 2002 as one of the first inducible lectins localized in the nucleus and the cytoplasm of plant cells (Chen *et al*., 2002). It has been demonstrated that the application of JA and its derivative methyl jasmonate (MeJA) induces lectin activity in the leaves of *N. tabacum* cv. Samsun NN. Lectin activity was detected in the JA/MeJA-treated leaves as well as in the systemic leaves, but not in the leaves of untreated plants (Lannoo *et al.,* 2007). Interestingly, only some *Nicotiana* species have been shown responsive to jasmonates by Nictaba synthesis, indicating that even closely related species may differ in specialized hormonal responses (Lannoo *et al.,* 2006a). Later on, Nictaba was also reported to be inducible in tobacco by pest herbivores including chewing insects (*Spodoptera littoralis* and *Manduca sexta*) and cell content feeders (*Tetranychus urticae*) presumably via activation of the jasmonate pathway (Lannoo *et al.,* 2007; Vandenborre *et al.,* 2009a, 2009b).

1.1.5.2 Nucleocytoplasmic localization of Nictaba

Nictaba is a homodimer consisting of two identical non-covalently linked subunits of 19 kDa. Analysis of the 165 AA sequence revealed that the protein lacks a signal peptide and comprises a putative nuclear localization signal (NLS) sequence (^{102}KKKK^{105}) suggesting that Nictaba is synthesized on free ribosomes in the cytoplasm and could be targeted to the nucleus (Chen *et al.,* 2002). Indeed, it was proven by immunocytochemistry that the tobacco lectin localizes in the nucleocytoplasmic compartment of tobacco parenchyma cells subjected to jasmonate treatment (Chen *et al.,* 2002). Further localization studies using native protein fusion with EGFP in transgenic *N. tabacum* BY-2 suspension cells and *N. benthamiana* plants confirmed localization of Nictaba in the nucleus and the cytoplasm (Delporte, 2013; Lannoo *et al.,* 2006b). However, even though the initial localization experiments with a mutant Nictaba protein impaired in the NLS indicated that nuclear localization is NLS-dependent, the latest results by Delporte (2013) using a Nictaba mutant with its NLS altered and a Nictaba mutant lacking carbohydrate-binding activity show that Nictaba transport to the nucleus might also rely on alternative mechanisms independent from both NLS and lectin activity.

1.1.5.3 Carbohydrate-binding activity of Nictaba

Nictaba promptly agglutinates red blood cells and this reaction can be most successfully inhibited with *N*-acetylglucosamine (GlcNAc) oligomers and certain glycoproteins of animal origin (e.g. thyroglobulin, asialofetuin and ovomucoid) (Chen *et al.,* 2002). Moreover, it has been shown by surface plasmon resonance that the lectin preferentially binds GlcNAc oligomers over monosaccharide GlcNAc and exhibits the highest affinity towards chitotriose ($GlcNAc_3$). Furthermore, more elaborate carbohydrate-binding studies using glycan arrays have demonstrated that Nictaba can also strongly interact with high-Man and bi-antennary complex N-glycans (Lannoo *et al.,* 2006b).

Finally, NMR analyses revealed that Nictaba has the highest affinity towards chitotriose as well as to the $Man_3GlcNAc_2$ core (present in both complex and high-Man *N*-glycans) (Gheysen, 2011). A three-dimensional model of Nictaba predicts a structure composed of two β-sheets of four and five antiparallel β-strands linked through extended loops and places the NLS readily accessible at the top of such a protruding loop. Based on this model and a mutational analysis performed by Schouppe *et al.* (2010), it turned out that tryptophan residues Trp15 and Trp22 are crucial for the lectin activity of Nictaba. Apparently, the sugar-binding activity of Nictaba is most probably mediated through the large electronegatively charged groove (established mostly by glutamic acid residues Glu138 and Glu145) and gets stabilized by aromatic stacking via Trp15 and Trp22 of the pyranose ring of the bound glycan.

1.1.5.4 Physiological role of Nictaba

Endogenous role in stress signaling

Based on the fact that Nictaba is induced after jasmonate application as well as after insect herbivory it is generally suggested to play an endogenous signaling role in stress physiology and defense responses of tobacco plants. Since it is a nucleocytoplasmic protein and a functional lectin with well-defined specificity, studies have been directed towards the identification of putative glycosylated interaction partners within the nucleocytoplasmic compartment of the plant cell with special focus on the nucleus. Initially, it was shown by far Western blot analysis that Nictaba binds to numerous *N*-glycosylated nuclear proteins from BY-2 cells in a carbohydrate-dependent manner (Lannoo *et al.,* 2006b). Strikingly, affinity chromatography and pull down assays (Delporte *et al.,*2014a; Schouppe *et al.,* 2011) as well as *in vivo* interaction studies including co-localization and molecular fluorescence complementation assays (Delporte *et al.,*2014a) demonstrated that Nictaba interacts with the core histone proteins through their *O*-GlcNAc modification. Altogether, based on its stress-inducible synthesis, localization in the nucleus as well as interaction with histone proteins the hypothesis was put forward that Nictaba functions in stress signaling by chromatin remodeling and regulation of defense-related gene expression (Delporte *et al. ,* 2014ab).

Role in plant defense

Since Nictaba expression is up-regulated after insect herbivory, it was speculated that the lectin might have an additional function in plant defense by directly affecting the predators. Interestingly, feeding experiments with the larvae of the pest insects *S. littoralis* and *M. sexta* performed on wild type (WT) tobacco plants as well as on transgenic *N. attenuata* plants ectopically over-expressing Nictaba or with reduced Nictaba expression, provided evidence that the tobacco lectin displays entomotoxic effects on Lepidopteran larvae (Vandenborre *et al.,* 2010). It is hypothesized that the insecticidal activity of Nictaba most probably relies on its lectin activity and interaction with glycoconjugates (e.g. chitin microfibrils) residing in the midgut of insects.

1.1.6 Nictaba-related proteins

1.1.6.1 Distribution of Nictaba-like proteins in plant kingdom

Extensive searches through publically available genome/transcriptome databases revealed that sequences homologous to Nictaba are widespread in the plant kingdom and are plant-specific, since no orthologs could be identified in other eukaryotes or in prokaryotes (Delporte *et al.,* 2015). Alignment of the AA sequence of Nictaba with the Nictaba domains of Nictaba-like proteins from different plant species (Schouppe *et al.,* 2010) revealed that the domain comprises several regions which are well preserved among the orthologs (Fig. 1.3A). Importantly, Trp residues required for the lectin activity of Nictaba are contained within those strongly conserved motifs. Apart from many tobacco species (Lannoo *et al.,* 2006a), Nictaba-related sequences with more than 40% sequence similarity with the Nictaba protein from *N. tabacum* have been identified in different dicots including *A. thaliana*, *Solanum lycopersicum* (tomato), *Solanum tuberosum* (potato), *Cucumis sativus* (cucumber), *Glycine max* (soybean), *Apium graveolens* (celery), *Lotus japonicas, Populus trichocarpa* (poplar), in monocots like *Hordeum vulgare* (barley) and *O. sativa* (rice), as well as in lower plants and *Physcomitrella patens* (Fig. 1.3B; Delporte *et al.,* 2015; Dinant *et al.,* 2003). With respect to the ubiquitous occurrence of proteins with Nictaba domain(s), it has been suggested that the tobacco lectin is the prototype of the family of Nictaba-like proteins (Delporte *et al.,* 2015). Nevertheless, despite such a wide distribution of Nictaba-related proteins, only few of them have been (partially) characterized until now.

1.1.6.2 Chimeric proteins containing a Nictaba domain

Interestingly, few orthologs are built up exclusively of the Nictaba domain, but most of them constitute chimeric proteins comprising one or more Nictaba domain(s) fused to unrelated N- and/or C-terminal domain(s) of either known or yet undefined function (Delporte *et al.,* 2015; Dinant *et al.,* 2003; Lannoo, 2007). Depending on the arrangement of the domains, a classification has been proposed grouping the Nictaba-related proteins into single (S-type) and multiple (M-type) domain proteins (Fig. 1.3; Delporte *et al.,* 2015). The S group type S0 orthologs contain the Nictaba domain only, while chimeric proteins preceded by an unrelated N-terminal domain are further subdivided into 6 different types (S1-S6) based on the variable length of this N-terminal motif and the presence or absence of an additional C-terminal sequence. Moreover, chimeric proteins with a known N-terminal domain are designated as type T (TIR-Nictaba), type A (AIG1-Nictaba), type P (protein kinase-Nictaba) and type F (F-box-Nictaba) proteins, and are following the subdivision analogous to the one for the S-type group based on the presence of additional N- or C-terminal motifs. Finally, the small M group of Nictaba-like proteins includes the type M1 protein built of a triple Nictaba motif with a medium long N-terminal sequence, as well as the type M2 protein built of a double Nictaba motif with an N-terminal F-box domain and a C-terminal sequence.

A

```
        10        20        30        40        50        60        70        80
MQGQWIAARD LSITWVDNPQ YWTWKTVDPN IEVAELRRVA WLDIYGKIET KNLIRKTSYA VYLVFKLTDN PRELERATAS
        90       100       110       120       130       140       150       160
LRFVNEVAEG AGIEGTTVFI SKKKKLPGEL GRFPHLRSDG WLEIKLGEFF NNLGEDGEVE MRLMEINDKT WKSGIIVKGF

DIRPN
```

B

Type	Species						
	Nicotiana tabacum	*Solanum lycopersicum*	*Arabidopsis thaliana*	*Physcomitrella patens*	*Selaginella moellendorffii*	*Oryza sativa*	*Cucumis sativus*
Single Nictaba domain							
Type S0	7	4	2	0	0	0	4
Type S1	1	5	2	0	0	1	4
Type S2	0	0	0	1	0	0	2
Type S3	0	1	2	0	0	1	3
Type S4	0	0	1	0	0	1	0
Type S5	2	2	0	0	0	2	0
Type S6	0	0	0	0	0	1	0
F-box-Nictaba							
Type F0	9	6	7	1	3	1	5
Type F1	5	2	9	0	1	4	4
Type F2	1	2	2	1	0	1	1
Type F3	0	0	1	0	0	0	0
TIR-Nictaba							
Type T1	0	6	4	0	0	0	0
AIG1-Nictaba							
Type A0	0	0	1	0	0	0	0
Protein kinase-Nictaba							
Type P0	0	0	0	0	0	1	0
Multiple Nictaba domains							
Type M1	0	0	0	0	0	1	0
Type M2	0	0	0	0	0	1	0

F-box domain; Nictaba domain; Short (<50 AA) N-terminal domain; TIR domain; Domain linker; Medium (50-100 AA) N-terminal domain; AIG1 domain; C-terminal domain; Long (>100 AA) N-terminal domain; Protein kinase domain

Fig. 1.3 Proteins with Nictaba domains. A, Amino acid sequence of the complete (165 AA) Nictaba protein (cDNA clone AF389848) from *Nicotiana tabacum* cv. Samsun NN. AAs highly conserved between different Nictaba-like proteins are marked in blue. Trp residues crucial for the lectin activity of Nictaba are indicated with red markers. B, Summary of the different types of Nictaba orthologs and their occurrence in some plant species (adapted from Delporte *et al.,* 2015).

TIR-Nictaba proteins

TIR (toll-interleukin 1 receptor) domains are commonly found in insects, mammals and plants as components of the specific resistance (R) proteins within the innate immune system (Burch-Smith TM and Dinesh-Kumar, 2007). Apart from the TIR domain, these R proteins in plants mostly consist of a nucleotide-binding (NB) domain and an LRR domain at the C terminus (TIR-NB-LRR proteins). So far, pathways involving TIR domain-containing proteins are not as well studied in plants as in other organisms, but it seems that they might have much more expanded functions, since they are suggested to play a role in pathogen detection and activation of downstream signaling processes but also in direct regulation of the expression of genes involved in defense responses.

AIG1-Nictaba proteins

Similar to the TIR domain, the AIG1 motif is also associated with plant defense responses. Its name refers to the resistance gene, called *avrRpt2*-induced gene 1, up-regulated in Arabidopsis plants upon infection with *Pseudomonas syringae* carrying the avirulence *Rpt2* gene (*avrRpt2*) (Cui *et al.,* 2013a; Reuber and Ausubel, 1996). So far, only one putative AIG1-Nictaba protein has been identified in Arabidopsis, but its function remains an open question.

Protein kinase-Nictaba proteins

Protein kinases are diverse enzymes catalyzing reversible protein phosphorylation in signal transduction pathways involved in plant responses to different environmental stresses (Lehti-Shiu and Shiu, 2012). The unique putative protein kinase-Nictaba found in rice has not been functionally characterized until now.

F-box-Nictaba proteins

In general, F-box proteins, composed of a conserved N-terminal F-box motif and a highly variable C-terminal domain, are known to play a crucial role in selective UPS-mediated protein degradation (Lannoo *et al.,* 2008; Skaar *et al.,* 2013). More details regarding F-box proteins, and their physiological roles in plants can be found in section 1.2.

Cucurbitaceae phloem lectins

Nictaba motifs have been also identified in the small group of Cucurbitaceae phloem lectins often called phloem proteins 2 (PP2s) (Beneteau *et al.,* 2010; Clark *et al.,* 1997; Dinant *et al.,* 2003). Similar to Nictaba, the PP2s are homodimers built of 17–25 kDa subunits and exhibit high affinity towards GlcNAc oligomers. In contrast to Nictaba, however, they are constitutively expressed and are typically localized in phloem exudates. Furthermore, despite relatively high sequence identity and similarity to Nictaba (33% and 51%, respectively), they lack an NLS and possess additional unrelated N- and C-terminal AA sequences (including the C-terminal cysteine rich pentapeptide necessary for inter-molecular disulphide bridge formation with the phloem protein PP1). Therefore, even though Nictaba and the Cucurbitaceae PP2 proteins are closely related, most probably they have a different function in plants.

1.2 Plant F-box proteins in the ubiquitin-26S proteasome system: judges between life and death

Proteins are continuously synthesized and degraded in all living cells and their half-lives may vary greatly ranging from just a few minutes or hours up to several weeks (Pratt *et al.,* 2002; Toyama and Hetzer, 2013). Importantly, protein turnover is not only required for the elimination of misfolded, denatured or damaged proteins, but also plays an essential role in adjusting the abundance of crucial regulatory proteins and enzymes. As such, protein degradation is a key post-translational event responsible for the regulation of a myriad of physiological processes and enabling rapid cellular response to internal as well as external signals.

A dominant proteolytic pathway in all eukaryotes involves the highly complex and tightly regulated machinery localized in the nucleus and cytoplasm of cells, further referred to as the ubiquitin (Ub)–26S proteasome system (UPS) (Vierstra, 2009). It is estimated that 10% of all intracellular proteins are short-lived and that most of them are subjected to proteasomal degradation. Therefore, it is not surprising that UPS has been at the center of research interest in the last years. The physiological relevance of protein turnover for all living organisms was recognized 10 years ago by awarding Aaron Ciechanover, Avram Hershko and Irwin Rose with the Nobel Prize in Chemistry for the discovery of Ub-mediated protein degradation (Giles, 2004).

1.2.1 The ubiquitin-26S proteasome system

1.2.1.1 Essentials of the UPS machinery

In the UPS, target proteins to be eliminated are specifically recognized and tagged by covalent ligation to Ub molecules for the ultimate degradation by the 26S proteasome (Smalle and Vierstra, 2004). Ub is a small 8.5 kDa protein built of 76 AA first discovered in 1975 which, as the name implies, is ubiquitously present in eukaryotes (Goldstein *et al.,* 1975; Vierstra, 2012). A chain of specifically arranged Ub moieties attached to a protein serves as a label directing the protein for proteasomal degradation (Thrower *et al.,* 2000). Target proteins can occur either in the nucleus and the cytoplasm, but can also be localized in the membranes facing the nucleocytoplasmic compartments or may be derived from the ER after retro-translocation into the cytoplasm (Hershko and Ciechanover, 1998). The process of protein tagging with the Ub chain relies on a sequential action of three enzymes: the Ub-activating enzyme (E1), the Ub-conjugating enzyme (E2) and the Ub-ligase enzyme (E3) (Fig. 1.4A; Smalle and Vierstra, 2004; Vierstra, 2009). First, the enzyme E1 forms a thioester bond between its Cys residue and the C-terminal Gly of an Ub molecule in an ATP-dependent manner and transfers the activated Ub to a Cys on the E2 enzyme. Then, E2 either donates Ub to the E3 or interacts with E3 and transfers the Ub molecule directly to the Lys residue on the target protein via an isopeptide bond (Berndsen and Wolberger, 2014).

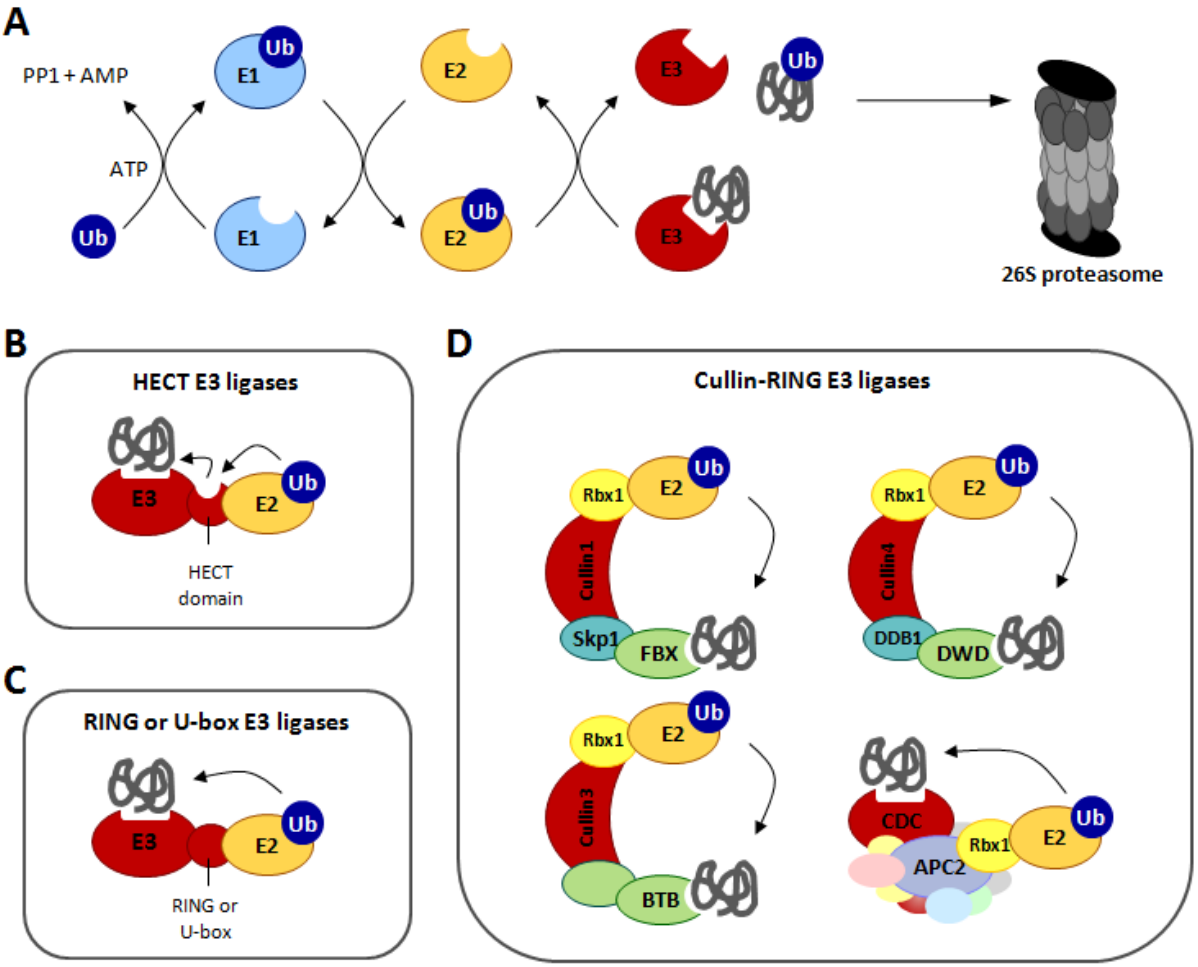


Fig. 1.4 Schematic representation of protein tagging for degradation via the UPS. A, Protein tagging with Ub is based on sequential action of three enzymes. First, Ub is activated by an E1 in an ATP-dependent manner. The activated Ub is transferred to an E2 and then is attached through an isopeptide bond to the target protein or to another Ub already present on the substrate. Protein labeled with a polyUb chain is directed for the degradation by the proteasome. B-D, Organization and mechanism for target recognition and ubiquitination by different types of E3s. B, The HECT E3 ligases are single polypeptides containing a HECT domain, which forms a thioester intermediate with Ub prior to protein ubiquitination. C, The RING or U-box E3 ligases are single polypeptides comprising a structurally related RING or U-box domain which binds the E2–Ub and promotes Ub transfer onto the target protein. D, The Cullin-RING ligases (CRL) are multisubunit E3s containing a Cullin, RING-box 1 (Rbx1) binding E2-Ub intermediate, and a variable substrate recognition module. The SCF-type E3s specifically bind the target via the F-box protein (FBX). The bric-a-brac–tramtrack–broad complex (BTB) E3s recognize their targets using BTB proteins. In the DNA damage-binding (DDB) E3s, WD40 domain-containing DWD proteins specifically bind the target protein. Finally, the anaphase-promoting complex (APC) E3s contain over 11 subunits including target-recognition module CDC.

This sequential process is repeated several times to attach more Ub moieties to each other through specific Lys residues (K48), ultimately giving rise to a polyUb chain necessary for the degradation of the substrate by the proteasome (Thrower *et al.,* 2000). The highly selective recognition and binding of the target protein is performed by an E3 ligase which, by interacting with both the E2-Ub complex as well as with the recognized target, mediates the transfer of a “death tag” to a specific protein. Based on their structure and mechanism of Ub transfer, E3 ligases are classified into four major types (Fig. 1.4B-D): HECT (Homologous to E6-associated protein C-Terminus), RING (Really Interesting New

Gene), U-Box and CRL (Cullin-RING Ligases) (Downes *et al.,* 2003; Hua and Vierstra, 2011; Mazzucotelli *et al.,* 2006; Petroski and Deshaies, 2005a; Stone *et al.,* 2005; Yee and Goring, 2009; Vierstra, 2009). The best-characterized are SCF complexes - the CRL-type E3s named after their components: Skp1 (S-phase kinase-associated protein 1), Cullin-1/CDC53 and F-box protein (Hua and Vierstra, 2011; Petroski and Deshaies, 2005a; Zheng *et al.,* 2002).

Remarkably, in comparison to other kingdoms, the UPS in plants constitutes an extremely complex and multifarious machinery illustrating the importance of selective protein degradation for plant physiology. In Arabidopsis, core UPS components are encoded by over 1600 genes, which would collectively express nearly 6 % of the proteome (Smalle and Vierstra, 2004; Vierstra, 2009). The hierarchical significance of each group of UPS constituents is well reflected by its diversity. In Arabidopsis e.g., only 2 out of those >1600 genes encode E1 enzymes (Hatfield *et al.,* 1997), at least 37 code for E2s (Kraft *et al.,* 2005), while most of them (>1400) encode putative E3 Ub-ligases responsible to confer specificity to the system (Mazzucotelli *et al.*, 2006).

1.2.1.2 Protein degradation by the 26S proteasome system

The 26S proteasome is an ATP-dependent multisubunit and multicatalytic protease complex of ~2.5 MDa present in the nucleocytoplasmic compartment (Kurepa and Smalle; 2008; Voges *et al.,* 1999; Wilk and Orlowski, 1980). It is structurally and functionally conserved in all eukaryotes. The proteasome fulfills the role of a cellular recycling machine, which specifically eliminates polyubiquitinated proteins by degradation into peptides. The 26S proteasome is composed of a cylindrical 20S core particle (CP) capped at either or both ends with the 19S regulatory particle (RP) (Finley, 2009). The CP consists of a stack of four heptameric rings surrounding the internal chamber. The rings are composed of two different types of subunits: structural α subunits and catalytic β subunits. The two outer rings of the cylinder consist of α subunits, which function as docking domains for RP. The inner rings built of β subunits possess three distinct proteolytic activities: chymotrypsin-like, trypsin-like and peptidylglutamyl-peptide hydrolyzing activity. It is the internal chamber of the CP lumen that harbors the active sites of the proteases and thus, this is the very place where the target proteins are enzymatically degraded into peptides. The opening to the CP lumen is narrow enough to restrict the entry only to the unfolded proteins (Groll and Huber, 2003). In turn, the RP of the proteasome serves as the selective gate to the internal proteolytic chamber (Kurepa and Smalle; 2008). It is composed of 19 individual subunits, out of which 10 form the lid and the other 9 the base. The lid RP Non-ATPase subunits (RPNs) include Ub receptors as well as deubiquitinating enzyme (DUB) and therefore are responsible for recognizing ubiquitinated substrates, removing the Ub chains and recycling Ub moieties. The base, binding directly to the α ring of the 20S CP, contains a ring of six RP Triple-A ATPases (RPTs) which unfold the target protein, open the entrance to the proteolysis chamber and transfer the target inside (Smith *et al.,* 2005).

In brief, a target substrate modified with Lys48-linked polyUb chain is specifically recognized by the Ub receptors in the RP lid, deubiquitinated by DUB and unfolded by ATPases localized in the RP base.

This unfolded protein is then translocated into the proteolytic chamber of the CP where it ultimately gets degraded into peptides. These peptides are further processed by other proteases into the constituent AAs reusable in the synthesis of new proteins.
With 23 genes encoding components of the 20S CP and 31 genes for 19S RP, plants assemble multiple proteasome variants with distinct compositions (Yang *et al.,* 2004). Some of these isoforms may have a particular cellular localization (either cytoplasm or nucleus), are differentially regulated or are uniquely designed to process specific protein substrates (Book *et al.,* 2009).

1.2.1.3 Physiological functions of the ubiquitin-26S proteasome system in plants

In the past few years, it was clearly demonstrated that protein modification with Ub as well as selective protein degradation by the proteasome represent an essential cellular machinery involved in most, if not all, aspects of plant physiology (Vierstra, 2009). Ubiquitination of numerous regulatory proteins controls a myriad of signaling and metabolic pathways. The UPS plays a role in plant developmental processes (Moon *et al.,* 2004, Schwechheimer and Calderón-Villalobos, 2004; Vierstra, 2009) including morphogenesis (Chae *et al.,* 2008; Coates *et al.,* 2006), self-incompatibility (SI) (Liu *et al.,* 2014), gameto- and embryogenesis (Wang *et al.,* 2013a), meristem formation (Di Giacomo *et al.,* 2013), organ size (Li and Li, 2014), trichome development (Patra *et al.,* 2013b), cell-cycle progression (Kim *et al.,* 2008) and circadian clock regulation (Cui *et al.,* 2013b). It is crucial for plant responses to hormones and for hormone biosynthesis, and in fact several Ub ligases are actually functioning as hormone receptors (Dharmasiri *et al.,* 2013; Kelley and Estelle, 2012). Moreover, the UPS constitutes a central regulatory mechanism in plant stress signaling upon diverse environmental challenges of both abiotic (Guo *et al.,* 2013; Stone, 2014; Zhang et al., 2014) and biotic origin (Alcaide-Loridan and Jupin, 2012; Dielen *et al.,* 2010; Dreher and Callis, 2007; Duplan and Rivas, 2014; Li *et al.,* 2014b; Magori and Citovsky, 2012; Marino *et al.,* 2012; Trujillo and Shirasu, 2010). The UPS is also involved in chromatin structure and epigenetics (Bourbousse *et al.,* 2012; Cao *et al.,* 2008a; Dhawan *et al.,* 2009; Gu *et al.,* 2009; Hu *et al.,* 2014; Zou *et al.,* 2014). In addition to its regulatory function, the UPS also performs a crucial housekeeping role of protein quality control by degradation of abnormal, misfolded and denatured proteins, thus preventing aggregate formation within the cell (Liu and Li, 2014).

1.2.2 F-box-proteins and their role in the ubiquitin-26S proteasome system

The SCF E3 Ub ligases are built up of four major constituents: Skp1, Cullin1/CDC53, an F-box protein and a Roc1/Rbx1/Hrt1 RING finger protein (Deshaies, 1999; Petroski and Deshaies, 2005a; Zheng *et al.,* 2002). Within SCF complexes, the structural Cullin-1 protein acts as a scaffold which ensures optimal exposure of the substrate for ubiquitination. It does so by bringing the catalytic module, that is the E2-Ub complex bound through Rbx1, in close proximity to the specifically recognized target bound to Cullin1 via the Skp1/F-box protein module (Cardozo and Pagano, 2004; Risseeuw *et al.,*

2003). It is the F-box protein which functions as an adaptor for the target protein and establishes the substrate specificity of the SCF complex (Skaar *et al.,* 2013). F-box proteins exhibit a typical bipartite structure containing a conserved N-terminal F-box domain of 40-50 AA first discovered in cyclin-F (Bai *et al.,* 1996; Craig and Tyers, 1999), and a highly variable C-terminal target-binding domain. While the F-box motif mediates protein-protein interactions with Cullin1-associated Skp1 and thus enables F-box protein incorporation into a functional SCF complex, the C-terminal domain recruits proteins destined for proteasomal degradation (Kipreos and Pagano, 2000; Skaar *et al.,* 2013). The F-box protein family is the largest protein superfamily known and numerous F-box proteins with different C-terminal motifs have been identified as SCF components. Based on the substrate-specific C-terminal domain, F-box proteins are categorized into three main classes: FBXW and FBXL families include F-box proteins with WD-40 and LRR domains, respectively, while members of the FBXO family contain other C-terminal domains, e.g. Kelch domains, Armadillo and tetratricopeptide repeats, zinc fingers, proline-rich or unknown motifs (Jin *et al.,* 2004). F-box proteins bind only to specific substrates which present a certain short, defined degradation signal referred to as the 'degron', enabling rapid and specific selection of the target protein (Fig. 1.5; Ravid and Hochstrasser, 2008). In general, members of FBXW and FBXL families are commonly implied in binding to phosphorylated targets through protein-protein interaction with the so-called phosphodegron (Fig. 1.5A; Skaar *et al.,* 2013). Nevertheless, for some F-box proteins target recruitment relies on different mechanisms. Recognition may be dependent on a cofactor (Fig. 1.5B; Lin *et al.,* 2006), can be limited by restricted access to the degron (Fig. 1.5C; D'Angiolella *et al.,* 2012) or by modification blocking the degron (Fig. 1.5D; Rossi *et al.,* 2013). Recruitment can also be controlled via localization of the substrate or the F-box protein (D'Angiolella *et al.,* 2012; Yao *et al.,* 2007) and may rely on inducible non-covalent degrons functioning as a 'molecular glue' (e.g. plant hormones; Fig. 1.5E; Sheard *et al.,* 2010; Tan *et al.,* 2007), or on covalent degron modifications other than phosphorylation e.g. glycosylation (Fig. 1.5F; Glenn *et al.,* 2008; Mizushima *et al.,* 2007; Yoshida *et al.,* 2002, 2003, 2005).
In fact, F-box proteins are themselves tightly regulated by the UPS (de Bie and Ciechanover, 2011). Proteasomal degradation of F-box proteins can be mediated either by an another E3 Ub ligase (An *et al.,* 2010; Bashir *et al.,* 2004; D'Angiolella *et al.,* 2012; Kim *et al.,* 2003; Klitzing *et al.,* 2011; Margottin‐Goguet *et al*., 2003; Wei *et al.,* 2004) or via the autoubiquitination mechanism in the absence of the specific substrate (Galan and Peter, 1999; Scaglione *et al.,* 2007; Yen and Elledge*,* 2008).

Interestingly, apart from their canonical function in mediating proteasomal degradation, some F-box proteins in yeast and mammals can play an SCF-independent role on their own or in association with Skp1 (Hermand, 2006; Jonkers and Rep, 2009). For instance, they can act as chaperones by preventing aggregation of aberrant glycoproteins (Nelson *et al.,* 2013; Yoshida *et al.,* 2007), as transcription cofactors (Smaldone *et al.,* 2004), or participate in cell cycle (Hermand *et al.,* 2003; Kitagawa *et al.*, 1999; Wang *et al.,* 2003), mitochondria distribution and morphology (Dürr *et al.,* 2006; Kondo-Okamoto et al., 2006), and recycling of endosome components (Galan *et al.,* 2001).

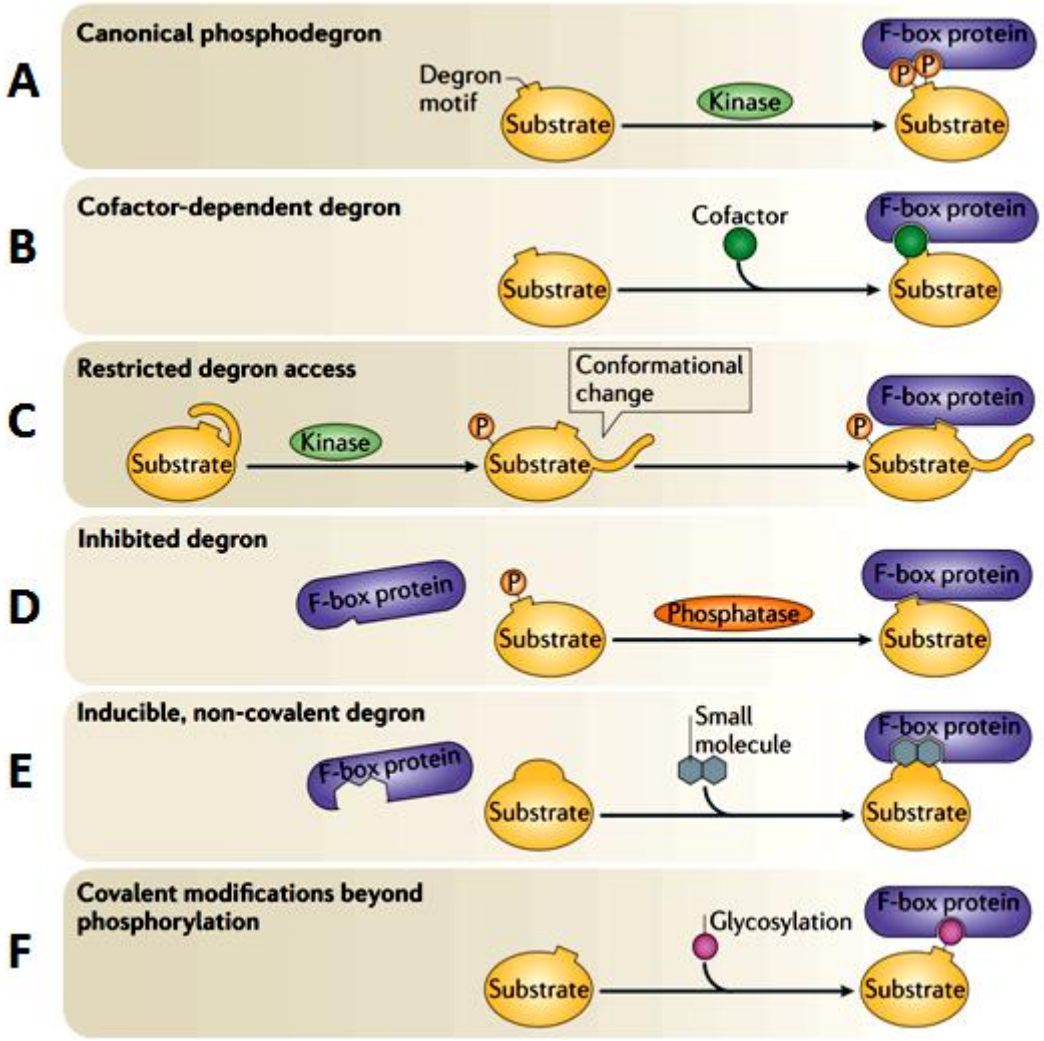


Fig. 1.5 Mechanisms of target recognition by F-box proteins (adapted from Skaar *et al.*, 2013). A, Recognition of phosphorylated targets through protein-protein interaction with the phosphodegron. B, Binding of the target dependent on a cofactor. C, Recognition of the target limited by restricted access to the degron. D, Degron recognition blocked by a modification. E, Binding dependent on an inducible non-covalent degron acting as a 'molecular glue'. F, Recognition of glycosylated targets via protein-carbohydrate interaction.

1.2.3 Involvement of F-box proteins in plant physiology

The F-box motif is highly widespread in plants and consequently, F-box proteins constitute one of the largest and most heterogeneous protein superfamilies known with a striking variability in C-terminal target-binding domains. Hundreds of putative representatives have been identified in different plant species including Arabidopsis (> 800 and >1350 members in *A. thaliana* and *A. lyrata,* respectively) (Gagne *et al.,* 2002; Hua *et al.,* 2011; Kuroda et al., 2002; Yang *et al.*, 2008), *M. truncatula* (>1100), *G. max* (>700) (Bellieny-Rabelo *et al.,* 2013), *O. sativa* (> 900) (Jain et al., 2007), *P. trichocarpa* (>400) and *Z. mays* (>400) (Hua *et al.,* 2011). For comparison, *Saccharomyces cerevisiae, Drosophila melanogaster* and human genomes encode only 20, 27, and 69 putative members of the F-box family, respectively (Skaar et al., 2009). Similarly, in plants multiple plant Skp1-like proteins have

been identified: e.g. there are 21 Arabidopsis-SKP1-like (ASK) proteins and 28 members in rice (Dezfulian *et al.,* 2012; Kong *et al.,* 2004, 2007; Kuroda *et al.,* 2012; Marrocco *et al.,* 2003; Zhao *et al.,*2003), while there is only a single representative in humans and in *S. cerevisiae* (Kipreos and Pagano, 2000). Based on the structure of the domain organization and on the type of C-terminal recruitment motif, the plant F-box superfamily has been subdivided in 42 families: apart from the well-characterized C-terminal domains like LRR, WD-40, Kelch, TPR, Tubby, RING finger or Armadillo, they may contain some other unique motifs which are often plant-specific, e.g. the FBD domain (Jain *et al.,* 2007; Kuroda *et al.,* 2002; Xu *et al.,* 2009). This enormous expansion of the class of F-box proteins in plants and the impressive variability of their C-terminal substrate-binding motifs, altogether with numerous Skp1-like proteins mediating F-box protein interaction with Cullin1, suggest that plants could theoretically assemble thousands of different SCF complexes targeting a wide collection of diverse proteins involved in a multitude of physiological processes (Vierstra, 2009). It is hypothesized that plants, being sessile organisms continuously challenged with a plethora of environmental signals, were forced to develop this highly sophisticated and tightly regulated machinery of post-translational control, in order to rapidly respond in a specific manner to different cues and stresses, which ultimately helps them to survive. Indeed, F-box proteins are associated with many biological events in plant development and stress signaling, including morphogenesis, hormone perception and signaling, cell cycle, circadian clock regulation, SI, senescence, responses to abiotic stress and plant-pathogen interactions (Guo *et al.,* 2013; Kelley and Estelle 2012; Lechner *et al.,* 2006; Magori and Citovsky, 2012; Marino *et al.,* 2012; Vierstra, 2009). Nevertheless, despite the large progress made during the past decade, only a small fraction of the putative F-box proteins identified in plants has been (partially) characterized (Table 1.2).

1.2.3.1 Phytohormone signaling

Plant hormones are endogenously synthesized small signaling molecules which regulate all aspects of plant growth, development and responses to environmental cues (Santner *et al.,* 2009). UPS-mediated protein degradation has been implicated in almost every signaling pathway by regulating the expression of genes specific to each hormone, including auxin, cytokinin, JA, gibberellin (GA), ethylene (ET), salicylic acid (SA), ABA, brassinosteroid (BR) and strigolactone (SL) (Dharmasiri *et al.,* 2013; Kelley and Estelle, 2012). Studies over the past decade have shown that in most of these pathways F-box proteins are the key signaling components acting as hormone receptors responsible for signal perception and transduction. Degradation substrates can be recruited to F-box proteins via degrons composed of both a small hormone molecule and a specific target protein sequence. The molecular mechanisms of this interaction rely on hormones which can either act as a 'molecular glue' filling the gaps between the F-box proteins and their substrates, extending the binding surface and stabilizing the interaction, or might cause an allosteric change leading to the exposure of interaction domains (Lumba *et al.,* 2010*;* Skaar *et al.,* 2013).

Table 1.2 Overview of plant and pathogen F-box proteins with demonstrated involvement in plant physiology.

Process	Species	F-box proteins	Target-binding domain	Confirmed/ putative target	References
Auxin signaling	*A. thaliana*	TIR1 AFB1-5	LRR	Aux/IAA	Calderón Villalobos *et al.,* 2012; Dharmasiri *et al.,* 2005a, b; Gray *et al.,* 2001; Greenham *et al.,* 2011; Hayashi, 2012; Kepinski and Leyser, 2005; Peer, 2013; Tan *et al.,* 2007;
JA signaling	*A. thaliana*	COI1	LRR	JAZ	Chini *et al.,* 2007; Sheard *et al.,* 2010; Thines *et al.,* 2007; Wasternack and Hause; 2013; Xie *et al.,* 1998; Xu *et al.,* 2002; Yan *et al.,* 2013;
GA signaling	*A. thaliana*	SLY1 SNE	GGF and LSL	DELLAs: GAI, RGA, RGL1-3	GA signaling
	O. sativa	GID2		SLR1	
Ethylene signaling	*A. thaliana*	EBF1-2 ETP1-2	LRR FBA	EIN3 EIL1 EIN2	An *et al.,* 2010; Gagne *et al.,* 2004; Guo and Ecker., 2003; Ji and Guo, 2013; Merchante *et al.,* 2013; Potuschak *et al.,* 2003; Qiao *et al.,* 2009;
ABA signaling	*A. thaliana*	TLP9 EDL3 DOR	Tubby domains Leucine-zipper N/D	N/D	Koops *et al.,* 2011; Lai *et al.,* 2004; Zhang *et al.,* 2008;
Strigolactone signaling	*A. thaliana*	MAX2/ORE9	LRR	BES1	Beveridge *et al.,* 1996; Drummond *et al.,* 2012; Jiang *et al.,* 2013; Smith and Li, 2014; Stirnberg *et al.,* 2002, 2007; Wang *et al.,* 2013b; Zhou *et al.,* 2013;
	O. sativa	D3		D53	
	P. sativum	RMS4		N/D	
	P. hybrida	MAX2a		N/D	
Root development	*A. thaliana*	ARABIDILLO1-2 CEG/SFL61 VBFs AUF1-2	Arm-repeats N/D LRR LRR	N/D	Coates *et al.,* 2006; Dong *et al.,* 2006; Schwager *et al.,* 2007; Zheng *et al.,* 2011
Floral development	*A. thaliana*	UFO	N/D	N/D	Chae *et al.,* 2008; Hepworth *et al.,* 2006; Ingram *et al.,* 1997; Ni *et al.,* 2004; Samach *et al.,* 1999;
	Antirrhinum majus	FIM	Kelch repeats		
Senescence	*O. sativa*	FBK12 D3	Kelch repeats LRR	SAMS1 D53?	Chen *et al.,* 2013; Woo *et al.,* 2001; Yan *et al.,* 2007;
	A. thaliana	MAX2/ORE9	LRR	BES1?	
Nodulation	*L. japonicus*	TML	Kelch repeats	N/D	Takahara *et al.,* 2013;
Cell cycle	*A. thaliana*	SKP2A SKP2B FBL17	LRR	E2FC DPB KRP1 KRP3-7	del Pozo *et al.,* 2002, 2006; del Pozo and Manzano, 2013; Jurado *et al.,* 2010; Kim *et al.,* 2008; Ren *et al.,* 2008b; Zhao *et al.,* 2012a;
Light signaling	*A. thaliana*	EID1 AFR	Leucine-zipper Kelch repeats	N/D N/D	Dieterle *et al.,* 2001; Harmon and Kay, 2003; Marrocco *et al.,* 2006;
Circadian clock regulation	*A. thaliana*	ZTL LKP2 FKF1	Kelch repeats	TOC1 PRR5 TOC1 PRR5 CDF1-2	Baudry *et al.,* 2010; Fornara *et al.,* 2009; Imaizumi *et al.,* 2003, 2005; Ito *et al.,* 2012; Kiba *et al.,* 2007; Kim *et al.,* 2003; Mas *et al.,* 2003; Nelson *et al.,* 2000; Sawa *et al.,* 2007; Schultz *et al.,* 2001; Somers *et al.,* 2000, 2004; Takase *et al.,* 2011;

Self-incompatibility	Solanaceae Plantaginaceae Rosaceae	SLFs	FBA	S-RNAses	Huang *et al.,* 2006; Kubo *et al.,* 2010; Li *et al.,* 2014c; Liu *et al.,* 2014; Qiao *et al.,* 2004a, b; Sijacic *et al.,* 2004; Sonneveld *et al.,* 2005; Takayama and Isogai, 2005;
Abiotic stress responses	*A. thaliana*	FBP7	N/D	N/D	Bu *et al.,* 2014; Calderón-Villalobos *et al.,* 2007; Chen *et al.,* 2008; Chen *et al.,* 2014; Li *et al.,* 2014d; Maldonado-Calderón *et al.,* 2012; Zhou *et al.,* 2014;
		At5g21040	WD40		
		MAX2/ORE9	LRR		
		PP2-B11	CRD		
	C. annuum	CaF-box	LRR		
	P. vulgaris	FBS1	N/D		
	T. aestivum	FBA1	AMN1		
Defense responses	*A. thaliana*	CPR1/CPR30	FBA	SNC1 RPS2	Cheng *et al.,* 2011; Gou *et al.,* 2009, 2012; He *et al.*, 2012; Kim and Delaney, 2002; Ralhan *et al.,* 2012; Wang *et al.,* 2014; Zaltsman *et al.*, 2010;
		COI1/coi1^{rsp}	LRR	N/D	
		SON1	FBA	N/D	
		VBF	CRD	VIP1	
	N. benthamiana *N. tabacum* *S. lycopersicum*	ACIF1 (ACRE189)	LRR	N/D	van den Burg *et al.,* 2008;
	O. sativa	DRF1	N/D		Cao *et al.,* 2008b;
Manipulation of host SCF machinery by plant pathogens	*A. tumefaciens*	VirF	N/D	VIP1 VirE2	Magori and Citovsky, 2012; Tzfira *et al*., 2001, 2004;
	R. solanacearum	GALA1,5,6,7	LRR	N/D	Angot *et al.*, 2006; Poueymiro and Genin, 2009;
	Poleroviruses Enamovirus	P0	N/D	AGO1	Baumberger *et al.,* 2007; Bortolamiol *et al.,* 2007; Csorba *et al.,* 2010; Pazhouhandeh *et al.,* 2006;
	Nanovirus	Clink	LxCxE	RBR	Aronson *et al.,* 2000; Lageix *et al.,* 2007;

Domains: CRD, carbohydrate-recognition domain; FBA, F-box-associated motif; GGF and LSL, domain name corresponds to the conserved AA residues; LOV, light-oxygen-voltage domain; LRR, leucine reach repeats; PAS, Per-Arnt-Sim domain; N/D, non-defined.

Auxin signaling

Auxins play a cardinal role in the regulation of plant growth and developmental processes ranging from embryogenesis to senescence (Peer, 2013; Woodward and Bartel, 2005). Auxin responsive genes in Arabidopsis are under the control of transcription factors called Auxin Response Factors (ARFs) which are negatively regulated by interaction with the complex of transcriptional repressors Aux/IAA (Auxin/Indole-3-acetic acid) and TOPLESS (TPL) co-repressor protein (Fig. 1.6; Szemenyei *et al.,* 2008). Under increased auxin concentration Aux/IAAs are polyubiquitinated and directed for proteasomal degradation through specific interaction with the F-box proteins TRANSPORT INHIBITOR RESPONSE1 (TIR1) or AUXIN SIGNALING F-BOX 1-5 (AFB1-5) associated within the SCF$^{TIR1/AFB1-5}$ complex (Gray *et al.,* 2001; Dharmasiri *et al.*, 2005b; Greenham *et al.,* 2011; Hayashi, 2012), ultimately leading to the derepression of auxin responsive genes. In this system, the F-box proteins TIR1/AFB1-5 were initially identified as auxin receptors (Dharmasiri *et al.,* 2005a; Kepinski and Leyser, 2005), whereas auxin serves as a 'molecular glue', enhancing and stabilizing the interaction between TIR1/AFBs and Aux/IAA proteins (Tan *et al.,* 2007). Nevertheless, it has recently been demonstrated that the assembly of the co-receptor complex consisting of both the TIR1/AFB and an Aux/IAA protein is crucial for efficient binding of auxin (Calderón-Villalobos *et al.,* 2012).

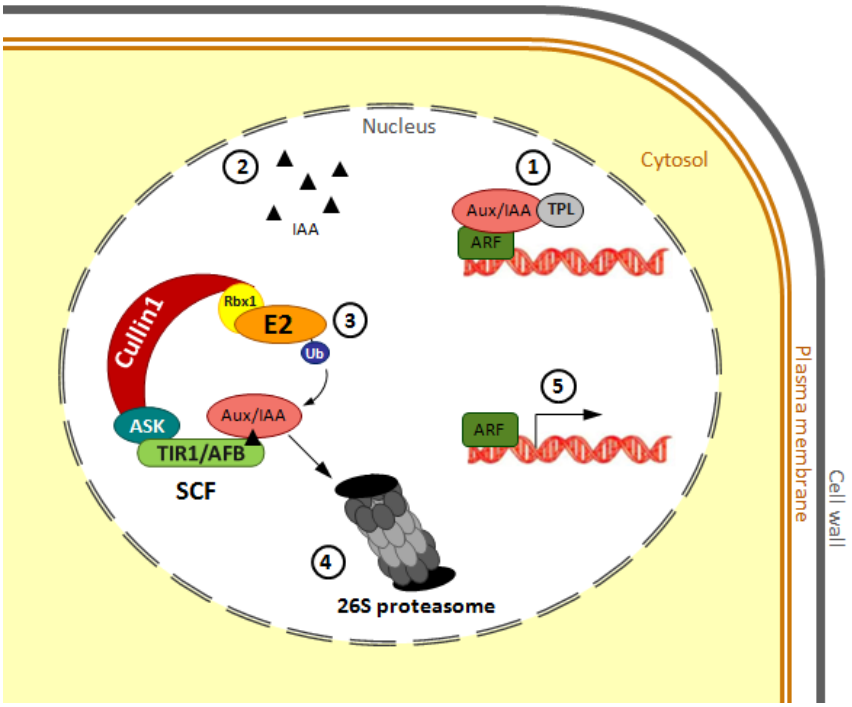


Fig. 1.6 Schematic representation of auxin signaling via the SCF-type E3 Ub ligase $SCF^{TIR1/AFB}$ (adapted from Dharmasiri *et al.,* 2013). (1) At low auxin levels, Aux/IAA and the TPL co-repressor interacts with the transcriptional factor ARF repressing gene transcription. (2) At high levels, auxin functions as molecular glue to promote the interaction between Aux/IAA and the TIR1/AFB proteins enhancing the ubiquitination of Aux/IAA repressors. (4) Ubiquitinated Aux/IAA is degraded through the 26S proteasome allowing (5) transcription of auxin-inducible genes.

Jasmonate signaling

Jasmonates, including JA and its derivatives, are plant signaling molecules mediating plant stress responses and are involved in plant growth and development (Wasternack and Hause, 2013). The mechanism of jasmonate signal perception and transduction in Arabidopsis strikingly resembles the one of auxin signaling (Pérez and Goossens, 2013). In association with other co-repressors (including TPL) the jasmonate ZIM-domain (JAZ) proteins block JA-mediated transcription by interaction with MYB/MYC transcription factors triggering JA-mediated transcription (Pauwels *et al.,* 2010; Shyu *et al.,* 2012). As a part of the SCF^{COI1} complex (Xie *et al.,* 1998; Xu *et al.,* 2002; Yan *et al.,* 2013) the F-box protein CORONATINE-INSENSITIVE1 (COI1) specifically recognizes the JAZ proteins and directs them for degradation by the proteasome, resulting in MYB/MYC transcription factors release and induction of JA-responsive gene transcription (Chini *et al.,* 2007; Thines *et al.,* 2007). Both COI1 and JAZ proteins function as co-receptors for JA-Ile, the physiologically active form of jasmonate which stabilizes the COI1-JAZ interaction via the molecular glue mechanism (Sheard *et al.,* 2010).

Gibberellin signaling

Gibberellins (GAs) are growth regulators controlling seed development and germination, but are also involved in organ elongation and flowering time (Hedden and Thomas, 2012). Similar to auxin and JA

signaling, GA-responsive genes are activated by degradation of DELLA transcriptional repressors (e.g. GAI in Arabidopsis and SLR1 in rice) which under low GA concentration, inhibit transcription by binding to PIF (PHYTOCHROME INTERACTING FACTORS) transcription factors (Hauvermale *et al.,* 2012; Hussain *et al.,* 2005; Ikeda *et al.,* 2001; Itoh *et al.,* 2005). Nevertheless, the GA signal perception is not performed by the F-box protein nor by its substrate, but by a separate soluble receptor called Gibberellin Insensitive Dwarf 1 (GID1). So far, this receptor has been identified in rice (Ueguchi-Tanaka *et al.,* 2005), barley (Chandler *et al.,* 2008) and three orthologs GID1a, GID1b, and GID1c have been also identified in Arabidopsis (Griffiths *et al.,* 2006; Nakajima *et al.,* 2006). The GA-bound GID1 receptor interacts with DELLAs and presumably causes their conformational change promoting recognition and binding to the corresponding SCF complex (Hirano *et al.,* 2010; Murase *et al.,* 2008; Willige et al., 2007). The identified SCF-associated F-box proteins targeting DELLAs for degradation by the proteasome include Arabidopsis SLEEPY1 (SLY1) and SNEEZY (SNE) (Ariizumi *et al.,* 2011; Dill *et al.,* 2004; Fu *et al.,* 2004; McGinnis *et al.,* 2003; Strader *et al.,* 2004) as well as rice GIBBERELLIN INSENSITIVE DWARF2 (GID2) (Gomi *et al.,* 2004; Sasaki *et al.,* 2003).

Ethylene signaling

Ethylene (ET) is a gaseous hormone regulating seed germination, senescence, abscission as well as plant responses to abiotic and biotic stresses (Merchante *et al.,* 2013). ET signaling is a very complex mechanism and SCF-associated F-box proteins are involved at several levels. Activation of ET-response genes in Arabidopsis is dependent on two positive transcriptional regulators: ETHYLENE INSENSITIVE 3 (EIN3) and EIN3-Like (EIL1). They are regulated through the interaction with two F-box proteins, EIN3-Binding F-box 1 (EBF1) and EBF2 (Gagne *et al.,* 2004; Guo and Ecker, 2003; Potuschak *et al.,*2003). In the absence of ET, $SCF^{EBF1/2}$ mediates ubiquitination and subsequent proteasomal degradation of EIN3/EIL1 (Fig. 1.7). At increased concentrations of the hormone, EBF1/2 themselves are subjected to proteasomal degradation, while consequently the levels of EIN3/EIL1 increase (An *et al.,* 2010). Nevertheless, stability of EIN3/EIL1 relies on an additional factor, the ETHYLENE INSENSITIVE2 (EIN2) protein. EIN2 is an ER transmembrane protein with a hydrophilic C-terminus at the cytoplasmic side (EIN2-CEND) which is cleaved off and translocated to the nucleus in response to the ET signal (Ji and Guo, 2013; Qiao *et al.,* 2012; Wen *et al.,* 2012). The EIN2-CEND prevents EIN3/EIL1 degradation, either by down-regulation of EBF1/2 expression (An *et al.,* 2010) or via a yet unidentified mechanism, leading to the activation of ET-response genes. Furthermore, EIN2 is also tightly regulated by multiple mechanisms including SCF-mediated protein degradation. ET is perceived via the membrane-bound receptors ETR1, ERS1, ETR2, ERS2 and EIN4 located in the ER which act as negative regulators of ET signaling. In the absence of the hormone, ET receptors activate another ER membrane-associated protein, the CONSTITUTIVE TRIPLE RESPONSE1 (CTR1), which is a Ser/Thr kinase phosphorylating EIN2, hereby blocking the cleavage and nuclear transport of EIN2-CEND (Ju *et al.,* 2012).

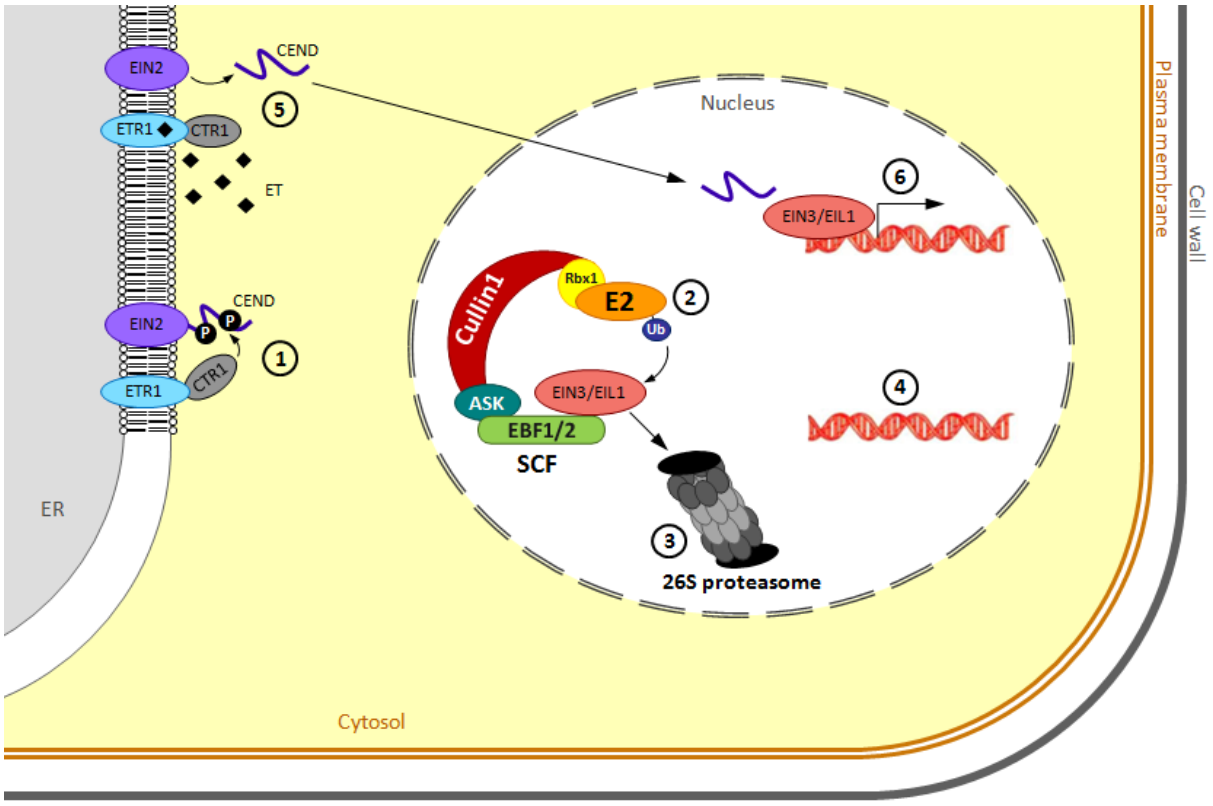


Fig. 1.7 Schematic representation of ET signaling via SCF-type E3 Ubligase $SCF^{EBF1/2}$ (adapted from Dharmasiri *et al.,* 2013). The ET receptor ETR1 and the EIN2 protein are localized in the ER membrane. (1) When there is no ET, the C-terminal end of EIN2 (EIN2-CEND) is phosphorylated through CTR1 and activated ETR1. (2) This allows the EIN3 transcriptional factor to interact with the F-box proteins EBF1/EBF2 enhancing the EIN3 ubiquitination (3) and degradation, (4) resulting in inhibition of gene transcription. (5) When ET binds to ETR1, ETR1 and CTR1 are deactivated inhibiting the phosphorylation of EIN2-CEND. This results in dephosphorylation of CEND and proteolytic cleavage. CEND is then translocated into the nucleus and will block the ubiquitination of EIN3. (6) Accumulation of EIN3 results in ET-induced gene transcription.

Upon ET binding the receptors become inactivated, what in turn inhibits CTR1 and permits positive regulation of downstream ET signaling through the EIN2 protein and EIN3/EIL1 transcription factors. Finally, the F-box proteins EIN2 TARGETING PROTEIN 1 and 2 (ETP1-2) have been demonstrated to trigger degradation of EIN2 in the absence of ET (Qiao *et al.,* 2009). In contrast, after ET signal recognition, ETP1-2 expression is decreased allowing the accumulation of EIN2.

Abscisic acid signaling

ABA plays an important role in seed and bud dormancy, embryo and seed development, reproduction as well as both abiotic (drought, salt, osmotic) and biotic stress responses. ABA signaling is rather complex and includes multiple steps requiring proteasomal degradation (Cutler *et al.,* 2010; Lee and Luan, 2012). A few F-box proteins in Arabidopsis are also involved, although their targets have not been established yet. F-box protein TLP9 has been suggested to participate in the ABA signaling pathway by conferring sensitivity to ABA during seed germination and early seedling development (Lai *et al.,* 2004). The EID1-LIKE PROTEIN3 (EDL3) is proposed to direct negative

regulators of ABA signaling for proteasomal degradation (Koops *et al.,* 2011). Furthermore, Drought Tolerance Repressor (DOR) inhibits the ABA-induced stomatal closure and probably regulates ABA biosynthesis in Arabidopsis under drought stress via the UPS (Zhang *et al.,* 2008).

Strigolactone signaling

Strigolactones (SLs) are the most recently identified class of phytohormones. They are synthesized in the root and play crucial roles in lateral shoot branching, root hair elongation and lateral root formation (Brewer *et al.,* 2012; Koltai, 2014; Smith and Li, 2014). Although the SL signaling mechanism is still unclear, it has been shown that the F-box proteins MORE AXILLARY BRANCHES2 (MAX2), DWARF3 (D3), RAMOSUS4 (RMS4) and MAX2a in Arabidopsis, rice, pea and petunia, respectively, are essential for the SL hormone response (Beveridge *et al.,* 1996; Drummond *et al.,* 2012; Jiang *et al.,* 2013; Stirnberg *et al.,* 2002, 2007). In addition, it has been demonstrated in Arabidopsis and rice that $SCF^{MAX2/D3}$ regulates SL-responsive gene expression by targeting transcription factors BES1/D53 for degradation (Jiang *et al.,* 2013; Wang *et al.,* 2013b; Zhou *et al.,* 2013).

1.2.3.2 Plant development and morphogenesis

F-box proteins play a pivotal role in almost every aspect of plant development by mediating plant hormone signaling. Nevertheless some F-box proteins seem to regulate developmental processes, such as root and flower development, without being directly implicated in hormone perception and signal transduction. Whereas two related F-box proteins ARABIDILLO-1 and ARABIDILLO-2 have been demonstrated to promote root branching in Arabidopsis (Coates *et al.,* 2006), the F-box protein CEGENDUO (CEG, AtSFL61), which is induced by auxin, decreases lateral root formation (Dong *et al.,* 2006). Additionally, four proteins belonging to the VIER F-BOX PROTEINE family (VFB; German for FOUR F-BOX PROTEINS) are required for lateral root formation and normal growth in general (Schwager *et al.,* 2007). Moreover, the auxin-responsive F-box proteins called AUXIN UP-REGULATED F-BOX PROTEIN1 and 2 (AUF1 and 2) are important positive regulators of root elongation presumably by targeting a positive effector controlling auxin movements and mediating the interplay between auxin and cytokinin signaling (Zheng *et al.,* 2011). These findings indicate that lateral root development might be tightly regulated by a complex crosstalk between degradation pathways mediated by different F-box proteins.

Two F-box proteins involved in flower formation and development have been described in plants: the UNUSUAL FLORAL ORGANS (UFO) from Arabidopsis and its ortholog FIMBRIATA (FIM) in *Antirrhinum*, which regulate multiple aspects of floral morphogenesis (Hepworth *et al.,* 2006; Ingram *et al.,* 1997; Ni *et al.,* 2004; Samach *et al.,* 1999). It has been demonstrated that UFO as a component of the SCF complex performs the role of a DNA-associated transcriptional co-factor in regulating *APETALA3* gene expression by promoting LEAFY transcription factor activity in a protein degradation-dependent manner (Chae *et al.,* 2008).

Also leaf senescence, seed size as well as grain number are under control of SCF-dependent degradation regulation. An F-box protein from rice, FBK12 (F-box protein containing a Kelch repeat motif), interacts with and targets S-ADENOSYL-L-METHIONINE SYNTHETASE1 (SAMS1) for proteasomal degradation (Chen *et al.,* 2013). Thereby, it delays senescence and germination and promotes increased seed size presumably via modulation of ET levels. In addition, the proteins MAX2/ORE9 and D3 F-box crucial for SL signaling in Arabidopsis and rice, respectively, have been shown to accelerate leaf senescence (Woo *et al.,* 2001; Yan *et al.,* 2007). Furthermore, MAX2 positively regulates photomorphogenesis and development, including optimization of seed germination, by modulating multiple hormone pathways other than SLs and in SL-independent manner (Shen *et al.,* 2012).

Recently, an F-box protein called TOO MUCH LOVE (TML) has been described as a key factor for proper organogenesis of root nodules during the final stage of autoregulation of nodulation in the model legume *Lotus japonicas*. It is suggested to suppress nodulation signaling prior to rhizobial infection by targeting the transcription factor associated with nodule formation for degradation (Takahara *et al.,* 2013).

1.2.3.3 Cell cycle

Protein degradation mediated by SCF complexes also regulates the cell cycle. Studies have shown that the *A. thaliana* S-Phase Kinase-Associated Protein 2A (SKP2A) is an auxin-binding F-box protein which controls proteolysis of the cell cycle repressors E2-promoter binding factor C (E2FC) and E2F dimerization partner B (DPB) (del Pozo *et al.,* 2002, 2006; del Pozo and Manzano, 2013; Jurado *et al.,* 2010). In the presence of auxin SKP2A mediates proteasomal degradation of E2FC and DPB, and thus promotes cell division. Interestingly, auxin also enhances UPS-driven degradation of SKP2A itself, acting as a factor preventing overfunction of the SCF mechanism. Two other F-box proteins, SKP2B and FBL17, are implicated in positive regulation of cell cycle progression by targeting cyclin-dependent kinase inhibitors, the so-called Kip-related proteins (KRPs), for SCF-mediated proteasomal degradation (Kim *et al.;* 2008; Ren *et al.,* 2008a; Zhao *et al.,* 2012a).

1.2.3.4 Light signaling and circadian clock regulation

F-box proteins are also involved in plant responses to light by regulation of the signaling pathway of the red and far-red light absorbing photoreceptor phytochrome A (phyA). While EMPFINDLICHER IM DUNKELROTEN LICHT (EID1) is a negative regulator of the phyA signaling pathway (Dieterle *et al.,* 2001; Marrocco *et al.,* 2006), the F-box protein ATTENUATED FAR-RED RESPONSE (AFR) is responsible for increased light sensitivity presumably by mediating degradation of a repressor of phyA signaling (Harmon and Kay, 2003). Until now, no targets have been found for EID1 nor for AFR.

More information is available regarding the control of photoperiod through proteasomal degradation. This process includes three photoreceptor F-box proteins containing an N-terminal LIGHT, OXYGEN OR VOLTAGE (LOV) domain (functioning as a blue-light-sensing domain) and a C-terminal Kelch repeat domain with an F-box motif in between. These proteins are called ZEITLUPE

(ZTL), FLAVIN-BINDING/KELCH-REPEAT/F-box 1 (FKF1) and LOV KELCH PROTEIN2 (LKP2) (Ito *et al.,* 2012). All three constitute the main regulators of the circadian clock in Arabidopsis by binding and targeting of the transcriptional regulators TIMING OF CAB EXPRESSION 1 (TOC1) as well as PSEUDO RESPONSE REGULATOR 5 (PRR5) implicated in photoperiodic flowering response for degradation (Baudry *et al.,* 2010; Kiba *et al.,* 2007 Mas *et al.,* 2003; Schultz *et al.,* 2001; Somers *et al.,* 2000, 2004). FKF1 additionally selects CYCLING DOF FACTOR 1 and 2 (CDF1-2) for degradation, which are transcriptional repressors of the CONSTANS crucial in circadian clock-dependent flowering (Fornara *et al.,* 2009; Imaizumi *et al.,* 2003, 2005; Nelson *et al.,* 2000; Sawa *et al.,* 2007). Interestingly, ZTL itself is also degraded via the 26S proteasome in a circadian phase-specific manner (Kim *et al.,* 2003), while FKF1 stability is modulated by both ZTL and LKP2 (Takase *et al.,* 2011). Altogether, ZTL, LKP2 and FKF1 F-box proteins cooperate through UPS-mediated control of transcriptional regulators in order to allow measurement of photoperiod and corresponding adaptation of flowering time (Ito *et al.,* 2012).

1.2.3.5 Self-incompatibility

In Solanaceae, Plantaginaceae and Rosaceae species a group of F-box proteins function in gametophytic SI, a process enhancing diversity by preventing from self-pollination and thus from inbreeding (Takayama and Isogai, 2005). In gametophytic SI, the pistil produces a set of polyallelic ribonucleases encoded in the S locus (S-RNases) which are transported into the growing tubes from both self and non-self pollen. In self pollen tubes, S-RNases are cytotoxic by degradation of RNA, ultimately leading to SI. In contrast, in the non-self tubes, S-RNases become specifically recognized and targeted for proteasomal degradation by corresponding polyallelic S-locus F-box proteins (SLFs) (Huang *et al.,* 2006; Kubo *et al.,* 2010; Li *et al.,* 2014c; Liu *et al.,* 2014; Qiao *et al.,* 2004a, b; Sijacic *et al.,* 2004; Sonneveld *et al.,* 2005). Consequently, a non-self pollen tube can grow further towards the ovary. The exact mechanism that allows differentiation between self and non-self recognition is not completely understood yet. Nevertheless, it is hypothesized that this phenomenon might rely on steric hindrance, high specificity of self/non-self pairs where interaction between self S-RNases and SLFs is impossible, or on the fact that self S-RNases activate auto-ubiquitination and subsequent degradation of self SLFs (Hua *et al.,* 2008; Kubo *et al.,* 2010).

1.2.3.6 Abiotic stress responses

Several F-box proteins have been associated with plant responses to abiotic stress factors. A cold- and heat-inducible F-box protein from Arabidopsis, FBP7, is apparently a key factor for protein synthesis under temperature stress (Calderón-Villalobos *et al.,* 2007). In turn, the Arabidopsis F-box protein encoded by At5g21040 negatively regulates inorganic phosphate starvation responses (Chen *et al.,* 2008). Recently, two Arabidopsis F-box proteins have been shown to play a role in plant responses to drought stress. The MAX2 protein, known for its role in SL signaling, involvement in leaf senescence and photomorphogenesis, has been demonstrated to be crucial for the plant response to drought and osmotic stress during seed germination and seedling growth (Bu *et al.,* 2014). The

mechanism by which MAX2 can control many diverse signaling pathways remains unclear although it is suggested that it might associate in many different SCF^{MAX2} complexes and target specific substrates involved in distinct signaling pathways at various developmental stages or in a tissue-specific manner (Bu *et al.,* 2014; Shen *et al.,* 2012). The other drought-related F-box protein is the phloem protein 2-B11 (AtPP2-B11) (Li *et al.,* 2014d). AtPP2-B11 expression is markedly induced upon drought treatment and its over-expression leads to drought hypersensitivity. It can interact with several Arabidopsis Skp1-like (ASK) proteins suggesting functionality in SCF complexes. AtPP2-B11 also binds a protein associated with desiccation, called AtLEA14 (late embryogenesis abundant protein 14), and decreases its levels under drought conditions. Also in wheat an F-box protein called TaFBA1 has recently been identified, which is upregulated upon water and salt stress as well as after ABA treatment (Zhou *et al.,* 2014). Its overexpression in tobacco conferred increased plant resistance to drought stress. In pepper, an F-box protein predominantly expressed in stems and seeds, called CaF-box, is up-regulated after treatment with various abiotic stress factors including cold and salt stress as well as after application of ABA and SA. It is therefore postulated that CaF-box fulfills an important role in pepper in the regulation of plant defense responses to abiotic stress (Chen *et al.,* 2014). Likewise, an F-box protein from bean, PvFBS1, is induced upon stress application including wounding, osmotic stress as well as after application of MeJA, SA and ABA. It is suggested to function in the general plant response to stress (Maldonado-Calderón *et al.,* 2012).

1.2.3.7 Plant-pathogen interactions

F-box proteins play an essential role in plant immune responses through their involvement in hormone pathways crucial for plant defense, i.e. JA, SA and ET signaling. However, numerous F-box proteins have been indentified which function in plant-pathogen interactions independently from hormone signaling regulation.

Plant F-box proteins in responses to pathogen infection

Similar to membrane-associated pattern recognition receptor (PRR) proteins recognizing pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) and damage-associated molecular patterns (DAMPs), also intracellular NLR (NB-LRR) immune receptors exist which detect specific pathogen effectors and trigger a downstream cascade of defense signaling (Muthamilarasan and Prasad, 2013; Wirthmueller et al., 2013). These resistance (R) proteins need to be tightly regulated and, apparently, they are often under the control of E3 Ub ligases (Duplan and Rivas, 2014). In Arabidopsis it has been demonstrated that two NLR R proteins, SNC1 (suppressor of npr1-1, constitutive 1) and RPS2 (resistant to *Pseudomonas syringae* 2), are specifically recognized and targeted for proteasomal degradation by the SCF-associated F-box protein CPR1/CPR30 (constitutive expresser of pathogenesis-related (PR) genes 1/30) preventing over-accumulation of these R proteins and excessive immune responses in healthy plants (Cheng et al., 2011; Gou et al., 2009, 2012). In turn, loss-of-function CPR1/CPR30 mutants exhibit constitutive activation of PR genes, accumulation of the defense hormone SA and enhanced resistance to *P. syringae* infection. Another example is the

COI1 F-box protein known for its role in JA-dependent signaling via the SCF^{COI1} complex. It appears that COI1 can also participate in JA-independent signaling by increasing Arabidopsis susceptibility to fungal infection or, in the $coi1^{rsp}$ allelic form, by regulating accumulation of the innate NB-LRR immune receptor (He *et al.*, 2012; Ralhan *et al.,* 2012). Moreover, the F-box-protein ACIF1 (Avr9/Cf-9-induced F-box 1; also called ACRE189) as part of the SCF^{ACIF1} complex, is required for cell death and SA-mediated defense responses activated upon pathogen recognition in tobacco and tomato plants (van den Burg *et al.*, 2008). ACIF1 homologs in *A. thaliana* (VFBs) are apparently associated with differential expression of stress-responsive genes. Another Arabidopsis F-box protein SON1 (suppressor of NIM1-1) is implicated in the negative regulation of defense signaling pathways against *Peronospora parasitica* and *P. syringae* independently of SA accumulation and systemic acquired resistance (SAR) (Kim and Delaney, 2002). Furthermore, the rice defense-related F-box protein (DRF1), inducible upon infection with the blast fungus *Magnaporthe grisea* and treatment with the chemical inducer of plant defense pathways benzothiadiazole, is a positive regulator of plant defense responses. Its overexpression in tobacco increases plant resistance to Tomato Mosaic Virus (ToMV) and *P. syringae* (Cao *et al.,* 2008b). Until now, the ACIF1, SON1 and DRF1 targets have not been identified.

Pathogen strategies to hijack host SCF machinery

Strikingly, pathogens have evolved strategies to counteract plant defenses and promote efficient infection by the synthesis of effectors or even F-box proteins able to reprogram the plant SCF machinery and to direct host cells to work for the benefit of the intruder (Alcaide-Loridan and Jupin, 2012; Magori and Citovsky, 2011b).

F-box proteins of pathogen origin

The first F-box protein identified in prokaryotes and demonstrated to function in eukaryotic host cells was the VirF protein from *Agrobacterium tumefaciens*, a pathogen causing crown gall disease by genetic transformation of plants (Schrammeijer *et al.*, 2001; Tzfira *et al.,* 2004). After *A. thaliana* transformation, *A. tumefaciens* translocates the VirF protein, single-stranded T-DNA coated with VirE2 proteins as well as other effectors into the host cell via the type IV secretion system (Magori and Citovsky, 2012). VirE2 interacts with the plant VIP1 protein (VirE2 interacting protein 1), facilitating the nuclear import of VirE2-coated T-DNA (Tzfira *et al.,* 2001). Then, by exploitation of the host UPS constituents in the nucleus, a functional SCF^{VirF} complex is formed (Schrammeijer *et al.*, 2001) required for proteasomal degradation of VirE2 and host VIP1 prior to T-DNA integration into the plant genome (Magori and Citovsky, 2012; Tzfira *et al.,* 2004). Interestingly, the Arabidopsis VIP1-binding F-box protein (VBF) targeting VIP1 for proteasomal degradation via the SCF^{VBF} complex is actually induced during infection with Agrobacterium, suggesting that VBF might be another host component manipulated by the pathogen to promote T-DNA incorporation (Zaltsman *et al.*, 2010; Wang *et al.,* 2014). Other F-box proteins of pathogen origin have been identified in *Ralstonia solanacearum*: four GALA type III secretion effector proteins comprise F-box domains and can interact with *A. thaliana* ASK1 and ASK2 (Angot *et al.,* 2006). At present, SCF^{GALA} host substrates have

not been discovered yet. Since a knockout of GALAs significantly diminishes *R. solanacearum* virulence, it can be concluded that these proteins are of crucial importance for the pathogen infection strategy (Poueymiro and Genin, 2009).

Also viruses have been demonstrated to encode F-box proteins and use them to hijack the host SCF system. The P0 proteins from the Polero- and Enamovirus are F-box proteins which associate with plant SCF components and mediate degradation of the ARGONAUTE1 (AGO1) protein crucial for the RNA-induced silencing complex (RISC) (Baumberger *et al.,* 2007; Bortolamiol *et al.,* 2007; Csorba *et al.,* 2010; Fusaro *et al.,* 2012; Pazhouhandeh *et al.,* 2006). Consequently, they suppress the host RNA silencing system and enhance viral infectivity. The F-box protein Clink (for cell cycle link) encoded by the Nanovirus interferes with cell cycling by interaction with retinoblastoma-related proteins (RBR) responsible for blocking cell-cycle progression. In this way Clink directs cells into DNA synthesis and thus promotes intensive replication of the viral genome (Aronson *et al.,* 2000; Lageix *et al.,* 2007).

Plant hormone analogs of pathogen origin

Apart from synthesizing their own F-box proteins, pathogens can also control the plant SCF machinery and cell signaling by production of hormones or hormone analogs which manipulate host F-box proteins. An excellent example of mimicry used by plant pathogens is the bacterial toxin coronatine produced by *P. syringae* (Geng *et al.,* 2012). Generally, plant defense responses against bacteria including *Pseudomonas* rely on the activation of the SA-dependent pathway (Pieterse *et al.,* 2012). Yet, coronatine structurally and functionally mimics the bioactive conjugate Ja-Ile responsible for activation of JA-mediated defense responses by interaction with an F-box protein COI1 and promoting SCF^{COI}-mediated proteasomal degradation of JAZ transcriptional repressors (Sheard *et al.,* 2010; Thines *et al.,* 2007). Surprisingly, coronatine turns out to be approximately 1000-fold more active than the plant Ja-Ile in binding COI1 and its substrates (Katsir *et al.,* 2008). Hence, it may induce the JA signaling pathway antagonistic to the SA pathway and as a consequence perturb plant defense reactions (Geng *et al.,* 2012; Pieterse *et al.,* 2012; Uppalapati *et al.,* 2007). *P. syringae* also interferes with plant auxin signaling by synthesis of IAA (Glickmann *et al.,* 1998) leading to enhanced virulence by modulating plant susceptibility to infection (Fu *et al.,* 2011a; Mutka *et al.,* 2013). Although the mechanism is still unclear, it is hypothesized that the IAA of pathogenic origin could be perceived by plants similarly to the endogenous hormone via the IAA receptor, i.e. the F-box protein TIR1, and subsequently activate auxin-regulated gene expression (Fu and Wang, 2011; Tan *et al.,* 2007). In turn, the necrotrophic fungal pathogen of rice, *Gibberella fujikuroi,* produces GAs. Apparently, GA impedes JA-mediated plant resistance to necrotrophs by promoting F-box protein-driven degradation of DELLA repressors and subsequent activation of the GA-signaling pathway (Navarro *et al.,* 2008).

1.2.4 Carbohydrate-binding F-box proteins

The majority of plant F-box proteins contain at their C-terminus protein-binding domains (including LRR and Kelch repeats) recognizing substrates based on protein-protein interactions (Gagne *et al.,* 2002; Hua *et al.,* 2011; Jain *et al.,* 2007; Kuroda *et al.,* 2002; Xu *et al.,* 2009; Yang *et al.,* 2008). Most often, they target specific phosphorylated targets via recruitment of phosphodegrons (Skaar *et al.,* 2013). Nevertheless, taking into account the high diversity in C-terminal motifs within the FBXO family, substrate recognition might depend on many different mechanisms and on other covalent modifications. Accordingly, a decade ago the first F-box proteins with a CRD have been described in mammals (Glenn *et al.,* 2008; Mizushima *et al.,* 2007; Yoshida *et al.,* 2002, 2003, 2005) and soon afterwards also plant-specific glycan-binding proteins have been identified (Lannoo *et al.,* 2008).

1.2.4.1 Mammalian sugar-binding F-box proteins

The sugar-binding F-box proteins (Fbs proteins) containing a C-terminal CRD in mammals form a small family of proteins which direct glycoproteins for UPS-mediated degradation via carbohydrate-protein interactions (Yoshida, 2007). So far, the family includes five related proteins Fbs/Fbg1-2 and Fbg3-5, which comprise a conserved G domain structurally homologous to the carbohydrate binding domains of the mammalian lectins galectin and PNGase F (Glenn *et al.,* 2008). All five Fbs members can be incorporated into an SCF complex (Glenn *et al.,* 2008; Yoshida *et al.,* 2002, 2003). Interestingly, they exhibit diverse carbohydrate binding activities: Fbs1 and Fbs2 specifically recognize the core chitobiose ($GlcNAc_2$) of high-Man *N*-glycans, Fbg4 and 5 more preferentially bind complex glycans, while Fbg3 is apparently deprived of lectin activity (Glenn *et al.,* 2008; Hagihara *et al.,* 2005; Mizushima *et al.,* 2004, 2007; Yoshida *et al.,* 2002, 2003). Interestingly, Yoshida and co-workers have demonstrated that Fbs1/2 as part of SCF complexes play an essential role in glycoprotein homeostasis by targeting misfolded or unassembled glycoproteins into the ERAD system (Yoshida *et al.,* 2003; Yoshida *et al.,* 2005; Yoshida and Tanaka, 2010). Eukaryotic cells have developed a mechanism of ER protein quality control preventing from such danger (Helenius and Aebi, 2004; Liu and Li, 2014). In the ER, nascent proteins are modified with a high-Man *N*-linked glycan facilitating proper protein folding and serving as a specific marker of the folding state. If folding fails, the $Man_{3\text{-}9}GlcNAc_2$ *N*-glycan on misfolded proteins is specifically recognized as a signal for degradation. Subsequently, these proteins get translocated from the ER into cytosol for UPS-mediated degradation (Hüttner and Strasser, 2012; Meusser *et al.,* 2005). Though the core $GlcNAc_2$ motif of *N*-glycans in correctly folded proteins is not accessible to lectins, the chitobiose core in denatured (unfolded) glycoproteins becomes more exposed. Accordingly, it has been shown that $SCF^{Fbs1/Fbs2}$ bind exclusively to denatured glycoproteins in the cytosol and act as E3 Ub ligases in the ERAD pathway (Yoshida, 2003; Yoshida *et al.,* 2005; Yoshida and Tanaka, 2010).

1.2.4.2 Plant F-box proteins with a lectin domain

Hitherto, no functional Fbs proteins targeting misfolded or incompletely assembled glycoproteins for ERAD have been described in plants. Nevertheless, evidence has been presented that plants express a small family of putative F-box proteins comprising a C-terminal lectin-like domain which could presumably recognize and bind glycans and/or glycoproteins in a carbohydrate-specific manner (Delporte *et al.,* 2015; Dinant *et al.,* 2003; Lannoo *et al.,* 2008; Van Damme *et al.,* 2008). The C-terminal domain of most of these proteins is homologous to Nictaba, the nucleocytoplasmic lectin from tobacco plants (Chen *et al.,* 2002; Lannoo, 2007) identified as the prototype of the Nictaba-related protein family as described above (Delporte *et al.,* 2015). Strikingly, Nictaba can bind high-Man and bi-antennary complex *N*-glycans and exhibits the highest affinity towards chitotriose as well as to the $Man_3GlcNAc_2$ core (Gheysen, 2011; Lannoo *et al.,* 2006b). Thus the specificity of Nictaba resembles that of the mammalian Fbs1 and Fbs2 proteins (Glenn *et al.,* 2008; Yoshida *et al.,* 2002, 2003). What is more, the three-dimensional model of Nictaba is characterized by a β-barreled structure similar to the C-terminal lectin domain of the mammalian Fbs1 protein (Mizushima *et al.*, 2004; Schouppe *et al,.* 2010). Based on these observations it is hypothesized that F-box proteins containing a C-terminal Nictaba-like domain are functional analogs of the mammalian Fbs proteins responsible for targeting misfolded or incompletely assembled glycoproteins for ERAD (Fig. 1.8; Lannoo *et al.,* 2008).

Apart from the Nictaba-related plant-specific F-box proteins, two putative Man-binding F-box proteins have been found in Arabidopsis which contain a C-terminal domain homologous to jacalin (Van Damme *et al.,* 2008), a lectin isolated from the seeds of jack fruit (*Artocarpus integrifolia*) (Sastry *et al.,* 1986).

Putative F-box proteins with a Nictaba domain are widespread in the plant kingdom

Detailed screening of different plant genome and transcriptome databases revealed that F-box proteins comprising a C-terminal Nictaba domain are well conserved and widespread in the plant kingdom (Delporte *et al.,* 2015; Lannoo *et al.,* 2008). Homologs have been found in dicots (*A. thaliana, C. sativus, S. lycopersicum, P. trichocarpa*), in monocots (*O. sativa*) as well as in lower plants (*S. moellendorffii*, *P. patens*). Nictaba domain-containing F-box proteins classified as F-type Nictaba-like proteins (Delporte *et al.,* 2015) are subdivided into 4 types depending on their domain organization. The F0 type represents the proteins which consist exclusively of an N-terminal F-box motif and a C-terminal Nictaba domain connected by a linker sequence. Putative F-box-Nictaba proteins which additionally contain an unrelated short (<50 AA) N-terminal domain but no C-terminal sequence are the type F1 proteins, the ones with a short N-terminal domain and a C-terminal sequence form the F2 group, while F-box-Nictaba proteins with a medium long (50-100 AA) N-terminal domain are of the F3 type.

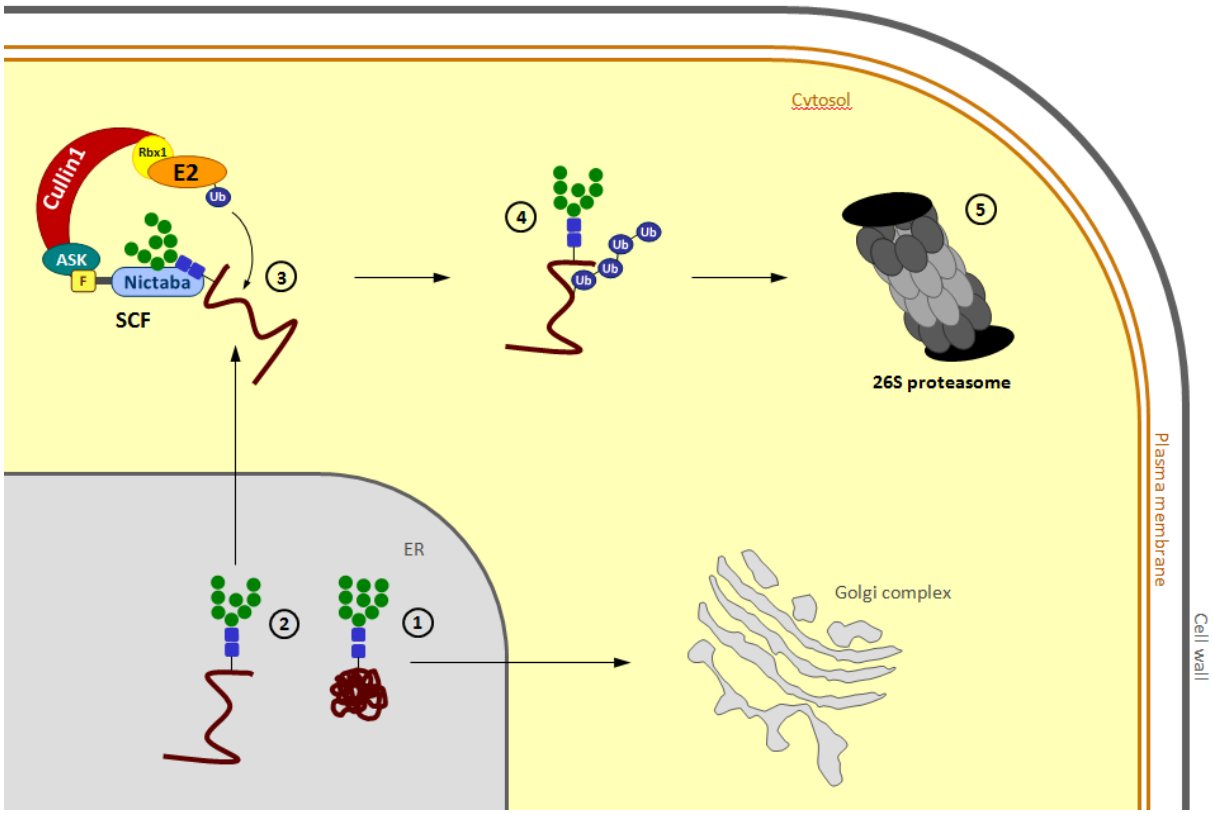
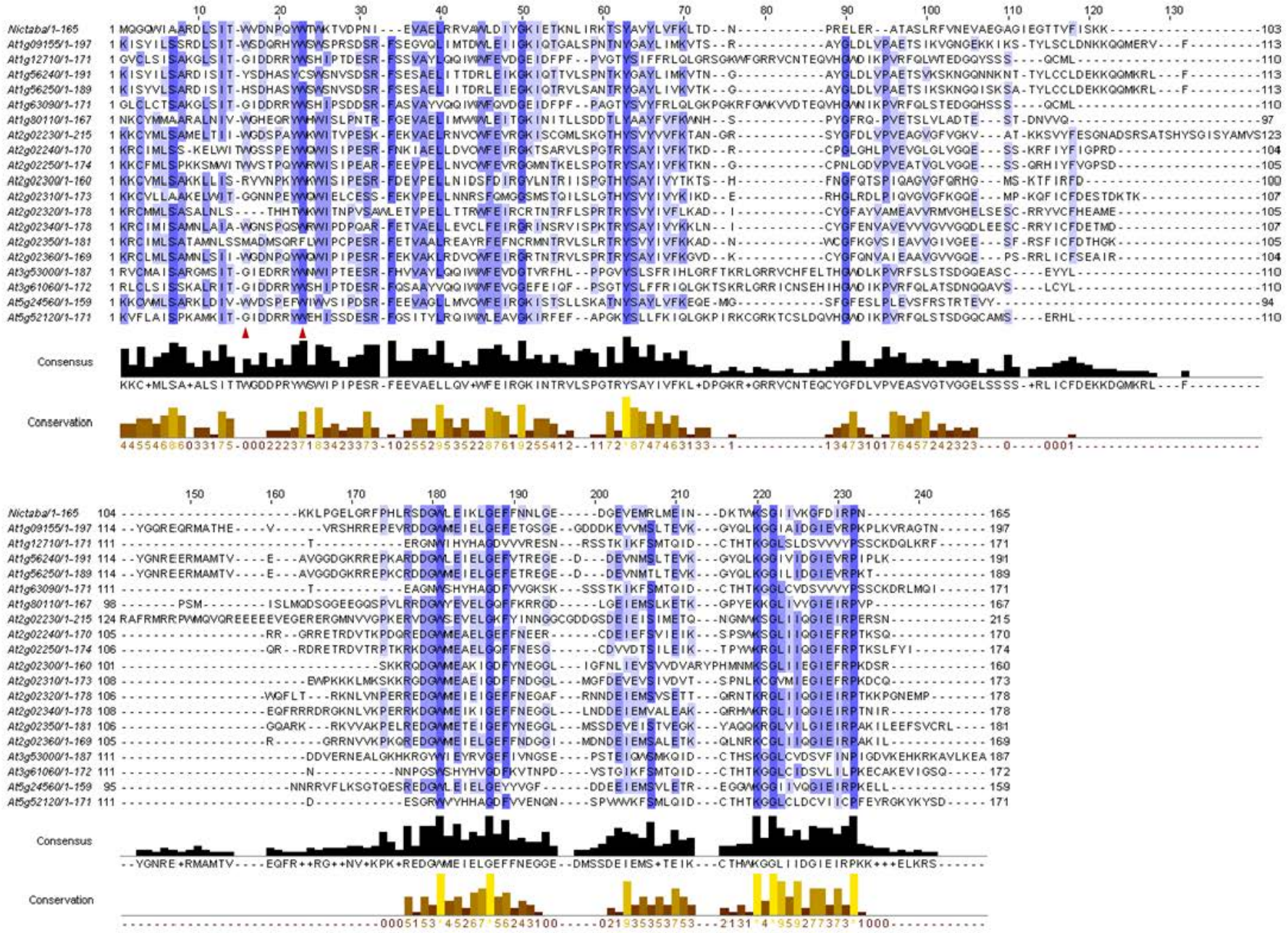


Fig. 1.8 Scheme representing the hypothetical role of plant-specific Fbs proteins belonging to the F-box-Nictaba family in ERAD. (1) A correctly folded protein will be transported from the ER to the Golgi complex for further processing. (2) A misofolded protein carrying the $Man_{3\text{-}9}GlcNAc_2$ *N*-glycan will be recognized and translocated from the ER into the cytosol. (3) In the cytosol, the *N*-glycan will be specifically bound by the Nictaba domain of an Fbs protein incorporated in the SCF complex and will become covalently labeled with Ub. (4) The protein tagged with a polyUb chain will be targeted for (5) degradation by the 26S proteaseome. Blue squares – GlcNAc residues, green circles – Man residues.

The highest number of Nictaba-related F-box protein-related sequences has been found in Arabidopsis which encodes approximately 30 homologs, although for some of them evidence at the level of cDNA or EST sequences is missing. Interestingly, the representatives show high AA sequence similarity in their F-box domain as well as in the Nictaba domain, corresponding to >90% and >40%, respectively. In addition, the general exon/intron structure is retained, where the first exon encodes the F-box domain, and the other two exons encode the Nictaba domain. As shown by the AA sequence alignment of the Nictaba (sequence encoded by AF389848) from *N. tabacum* cv. Samsun NN with Nictaba domains of Nictaba-related F-box proteins from *A. thaliana* (Fig. 1.9), the regions previously shown as conserved among Nictaba homologs from different plant species (Schouppe *et al.,* 2010; Fig. 1.3A), are strongly preserved in all sequences analyzed. Also, in most of the protein sequences, the AAs crucial for lectin activity of Nictaba (Trp15 and Trp22; Schouppe *et al.,* 2010) are well conserved (although Trp15 shows lower degree of conservation), suggesting that most likely these Nictaba-related sequences represent functional carbohydrate-binding proteins. Nevertheless the conservation of a few AA does not allow to predict the carbohydrate-binding specificity of a protein, since other AA outside the glycan-binding site will also play a role.


Consensus
Conservation
Consensus
Conservation

◀ **Fig. 1.9** Multiple sequence alignment of the amino acid sequences of of the complete (165 AA) Nictaba protein (cDNA clone AF389848) from *N. tabacum* cv. Samsun NN and of Nictaba-related F-box proteins from *A. thaliana*. The multiple sequence analysis was performed using the Clustal Omega sequence analysis tool (http://www.ebi.ac.uk/Tools/msa/clustalo; Sievers *et al.,* 2011) and was visualized using Jalview (http://www.jalview.org). Nictaba and AGI names of the Nictaba-related F-box proteins from Arabidopsis, together with the corresponding length of the Nictaba domains, are indicated in front of the sequences. AAs crucial for lectin activity of Nictaba (Schouppe *et al.,* 2010) are indicated with red markers. The consensus histogram shown below the alignment presents the percentage of the modal residue per column, where a '+' symbol indicates that the modal value is shared by more than one residue. The conservation histogram presents a quantitative alignment annotation measured as a numerical index reflecting the conservation of physico-chemical properties for each column of the alignment (Livingstone and Barton, 1993). Conserved columns score highest and the next most conserved columns contain substitutions to AAs of the same physico-chemical class. Increased conservation is visualised on the alignment as higher intensity of blue shading of the columns.

As shown by the phylogenetic tree reflecting the evolutionary relationships between the F-box proteins containing a C-terminal Nictaba domain from *A. thaliana* (Fig. 1.10), proteins encoded by genes located in the same chromosomal regions are clustered together. This indicates that these Nictaba domains are closely related and suggests that their expansion is associated with tandem gene duplication. Indeed, it has been reported that plant F-box genes often form tandem arrays in the same chromosomal regions and that extensive gene duplications have contributed to the evolution of many subsets of the F-box gene superfamily (Hua *et al.,* 2011; Jain *et al.*, 2007; Yang *et al.,* 2008; Xu *et al.,* 2009).

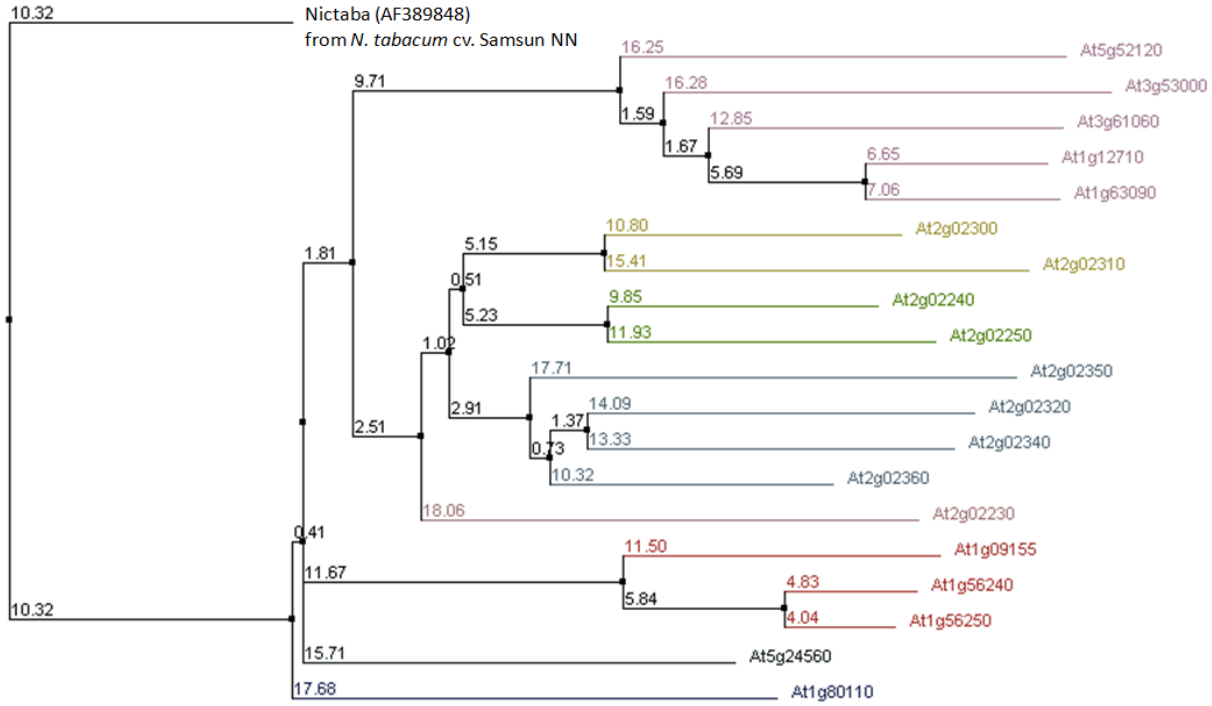


Fig. 1.10 Phylogenetic analysis of Nictaba-related F-box proteins from Arabidopsis showing the evolutionary relationship between the proteins. Phylogenetic tree was constructed with the AA sequences of the Nictaba domains of Nictaba-related F-box proteins from Arabidopsis aligned with the Clustal Omega sequence analysis tool as shown in Fig. 1.9 (http://www.ebi.ac.uk/Tools/msa/clustalo; Sievers *et al.,* 2011). The phylogenetic tree was generated using the neighbor-joining method and was visualized using Jalview (http://www.jalview.org). Numbers at the nodes and the lengths of the branches indicate the relative evolutionary distance.

Physiological role in plant stress signaling and involvement in the UPS

Both Nictaba from tobacco as well as some jacalin-related lectins belong to the group of nucleocytoplasmic plant lectins which are characterized by their stress-inducible expression and as such are hypothesized to play an important role in plant stress signaling (Lannoo and Van Damme, 2010). Indeed, Nictaba is up-regulated after jasmonate application as well as after insect herbivory. Furthermore, Nictaba interacts with the core histone proteins through their *O*-GlcNAc modification (Delporte *et al.,* 2014a; Schouppe *et al.,* 2011) suggesting a role in the regulation of defense-related gene expression (Delporte, 2013). Likewise, also the nucleocytoplasmic jacalin-related proteins have been shown to be involved in diverse plant stress responses (Al Atalah *et al.,* 2014a,c; de Souza Filho *et al.,* 2003; Moons *et al.,* 1997b; Qin *et al.,* 2003; Shinjo *et al.,* 2011). Consequently, in view of the general association of F-box proteins with a multitude of different developmental and stress-related processes as well as the presumed role of nucleocytoplasmic plant lectins in stress signaling, it is tempting to speculate that plant-specific F-box proteins with a C-terminal lectin domain might not only be involved in the elimination of unfolded proteins via the ERAD pathway, but could also play a signaling role in the nucleus and cytoplasm of plant cells by recognizing specific glycans or glycoproteins.

Table 1.3 gives an overview of the plant-specific F-box proteins containing a C-terminal lectin domain identified in *A. thaliana* with special focus on the information available regarding their putative physiological role in plants, i.e. potential interactors, expression profile and subcellular localization. Most of the proteins are predicted to localize in the nucleus and/or the cytoplasm, and three of them (F-box-Nictaba (Lannoo, 2007), VBF (Wang *et al.,* 2014; Zaltsman *et al.*, 2010) and SKIP12 (Li *et al.,* 2014d) encoded by *At2g02360*, *At1g56250* and *At1g80110*, respectively) have already been experimentally confirmed as nuclear or nucleocytoplasmic proteins. About half of the putative carbohydrate-binding F-box proteins have already been demonstrated to interact with at least one ASK protein, indicating that they can be associated into SCF complexes. These plant Fbs-like proteins preferentially associate with ASK1-2 and ASK11-12, which generally show a broad interaction potential and are quite commonly bound by many diverse Arabidopsis F-box proteins (Dezfulian *et al.,* 2012; Gagne *et al.,* 2002; Kuroda *et al.,* 2012; Risseeuw *et al.,* 2003; Takahashi *et al.,* 2004). Only a few plant Fbs proteins can also assemble with other ASKs. Almost all proteins for which expression information is available, are stress-inducible predominantly by abiotic but also after biotic challenges. Interestingly, the majority of these F-box proteins are particularly expressed in floral organs as well as in pollen and seeds. Strikingly, such flower- , pollen- and seed-specific expression pattern is also characteristic for many of the ASK proteins including those demonstrated as binding partners for plant Fbs proteins (Dezfulian *et al.,* 2012; Kuroda *et al.,* 2012; Marrocco *et al.,* 2003; Takahashi *et al.,* 2004; Zhao *et al.,* 2003).

Aside from ASKs, SKIP12, VBF as well as At2g02300 have already been demonstrated to interact also with other proteins. At2g02300 apparently can bind the yet uncharacterized protein LSU1 (low sulfur protein 1) and the transcription factor TCP14 (Arabidopsis Interactome Mapping Consortium, 2011), while two other proteins have been studied in more detail and were already briefly mentioned in section 1.2.3 with respect to their role in plant responses to (a)biotic stresses. The first one, called VIP1-binding F-box protein (VBF) encoded by *At1g56250,* is involved in plant-pathogen interactions during Arabidopsis infection with *A. tumefaciens* (Wang *et al.,* 2014; Zaltsman *et al.*, 2010). VBF regulates gene expression by targeting the VIP1 transcription factor for proteasomal degradation via the SCF^{VBF} complex (Zaltsman *et al.*, 2010). Apparently, VIP1 interacts with pathogen virulence factor VirE2 and thereby is exploited by the bacterium for nuclear import of its VirE2-coated T-DNA (Tzfira *et al.,* 2001). After fulfilling this role it is targeted for degradation altogether with VirE2 by the pathogen F-box protein VirF, allowing T-DNA uncoating and incorporation into the plant genome (Magori and Citovsky, 2012; Schrammeijer *et al.*, 2001; Tzfira *et al.,* 2004). Since VBF is induced during infection with Agrobacterium it has been suggested that it could be hijacked by the pathogen to promote T-DNA integration by even more efficient uncoating via degradation of the VIP1-VirE2 complex (Zaltsman *et al.*, 2010). However, recent findings indicate that another pathogen virulence factor, VirD5, actually competes with VBF for binding to VIP1 in the nucleus, preventing from too rapid degradation of the VirE2-VIP1 coat proteins by the host UPS (Wang *et al.,* 2014).
The other F-box protein with a Nictaba domain which has been partially characterized is the Arabidopsis phloem protein 2-B11 (AtPP2-B11) encoded by *At1g80110*. It was shown to negatively regulate plant responses to drought stress. AtPP2-B11 expression is significantly up-regulated after drought treatment and its over-expression leads to drought hypersensitivity during seed germination and in mature plants. AtPP2-B11 also binds a protein associated with desiccation, named LEA14 (late embryogenesis abundant protein 14) and decreases its levels under drought conditions. Furthermore, AtPP2-B11 over-expression results in altered gene expression of several abiotic stress-related markers (Li *et al.,* 2014d).

Apart from the latest experimental evidence for the role of VBF and AtPP2-B11 in plant stress responses, F-box proteins with a C-terminal lectin-like domain are poorly studied. In fact, none of these proteins has been confirmed as a functional lectin. In view of the fact that glycosylation is recently emerging as a highly complex coding system of crucial importance for cell signaling (Rüdiger and Gabius, 2009; Pilobello and Mahal, 2007), functional characterization of the plant carbohydrate-binding F-box proteins and unraveling their presumed role in glycoprotein degradation via the UPS might shed a new light on the cellular mechanisms underlying plant development and stress physiology.

Table 1.3 Overview of F-box proteins with a lectin domain in *A. thaliana.*

CRD	AGI name	Other names	Confirmed/ putative target(s)	Other confirmed interactors	Biological process	Expression profile		Location	References
						Tissue(s) with highest expression levels	Inducing factor(s)		
Nictaba	At1g09155	PP2-B15	N/D	N/D	N/D	stamen and petals	salt and osmotic stress (in roots); BS inhibitor	PM > ER > G > V [a]	-
	At1g12710	PP2-A12	N/D	N/D	N/D	rosette and cauline leaves, guard cells	cold stress	N > C [a]	-
	At1g56240	PP2-B13	N/D	N/D	N/D	N/D	N/D	PM > C > G > ER [a]	-
	At1g56250	PP2-B14 VBF	VIP1 [c]	ASK1, ABCI21, CW7	plant-pathogen interactions	N/D	*A. tumefaciens, E. coli*	PM > G, ER > V > C [a] N [b]	Arabidopsis Interactome Mapping Consortium, 2011; Wang *et al.,* 2014; Zaltsman *et al.*, 2010;
	At1g63090	PP2-A11 SKIP10	N/D	ASKs: 1-2, 11-12	N/D	rosette and cauline leaves	cold stress and wounding	C [a]	Kuroda *et al.,* 2012; Risseeuw *et al.,* 2003;
	At1g80110	PP2-B11 SKIP12	LEA14?	ASKs: 1-2, 7, 18-19	drought stress response	guard cells, trichomes, mature pollen	drought, cold, salt and osmotic stress; ABA, MeJA and cycloheximide; virulent *P. syringae*	N [ab] > C [ab]	Li *et al.,* 2014d; Risseeuw *et al.,* 2003;
	At2g02230	PP2-B1; SKIP21	N/D	ASKs: 1-4, 11-14	N/D	seeds and sepals, trichomes	salt (in roots); cycloheximide	N > C [a]	Risseeuw *et al.,* 2003; Kuroda *et al.,* 2012;
	At2g02240	MEE66	N/D	N/D	embryo development	N/D	N/D	C > N [a]	Pagnussat *et al.,* 2005;
	At2g02250	PP2-B2	N/D	N/D	N/D	sepals and mature pollen	SA; cold, drought, wounding, osmotic, genotoxic and oxidative stress (in roots)	C > N [a]	-
	At2g02300	PP2-B5	N/D	ASK2, LSU1, TCP14 [c]	N/D	petals	cold, osmotic, and genotoxic stress (in roots)	C [a]	Arabidopsis Interactome Mapping Consortium, 2011;
	At2g02310	PP2-B6	N/D	N/D	N/D	roots of young seedlings	cycloheximide; drought and salt stress (in roots); bacteria and bacterial elicitor; MeJA	N > C [a]	-
	At2g02320	PP2-B7	N/D	ASKs: 1-2, 11-12	N/D	N/D	N/D	M [a]	Kuroda *et al.,* 2012;
	At2g02340	PP2-B8	N/D	N/D	N/D	uniformly expressed	cycloheximide; cold and salt (in roots);	M > C >N [a]	-
	At2g02350	PP2-B9 SKIP3	N/D	ASK1	N/D	mature pollen	heat stress	P > C [a]	Farrás *et al.,* 2001;

	At2g02360	PP2-B10 F-box-Nictaba	N/D	ASKs: 1-2, 11-12	N/D	senescing leaves, cauline leaves, sepals and petals	heat stress, UV-B; photosynthesis and BS inhibitors, SA	N [ab]	Arabidopsis Interactome Mapping Consortium, 2011; Lannoo, 2007; Takahashi *et al.,* 2004;
	At3g53000	PP2-A15	N/D	N/D	N/D	seeds and mature pollen	cold stress	C [a]	-
	At3g61060	PP2-A13 SKIP9	N/D	ASKs: 1-2, 4-5, 11, 13; HSP15.7 HSP17.8	N/D	2nd internode, stamen, guard cells	cold and osmotic stress; ABA	C [a]	Arabidopsis Interactome Mapping Consortium, 2011; Gagne *et al.,* 2002; Kuroda *et al.,* 2012; Risseeuw *et al.,* 2003;
	At5g24560	PP2-B12	N/D	N/D	N/D	mature pollen	not affected	C [a]	-
	At5g52120	PP2-A14 SKIP13	N/D	ASKs: 1-2, 9, 11, 14, 19	N/D	cotyledons, first rosette leaves, roots of young seedlings, guard cells	MeJA; wounding	C [a]	Arabidopsis Interactome Mapping Consortium, 2011; Risseeuw *et al.,* 2003;
Jacalin	At3g59590	-	N/D	N/D	N/D	N/D	N/D	E [a]	-
	At3g59610	-	N/D	N/D	N/D	mature pollen, seeds and siliques,	BL, ACC, MeJA	N > C [a]	-

Since only a few of these proteins have been (partially) characterized until now, most expression data have been retrieved using the Arabidopsis eFP browser tool gathering the accessible gene expression information derived from different microarray analyses (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), while subcellular localization has been determined using the SUBA3 database combining manual literature curation, fluorescent protein visualization and protein-protein interaction datasets with subcellular targeting calls from 22 prediction programs ((http://suba.plantenergy.uwa.edu.au/; Tanz *et al.,* 2013).

N/D - non-defined; C – cytoplasm, E – extracellular space, ER – endoplasmic reticulum, G – Golgi, M – mitochondrion, N – nucleus, P – peroxisome, PM – plasma membrane, V – vacuole; [a] - predicted using SUBA3 database (http://suba.plantenergy.uwa.edu.au/; Tanz *et al.,* 2013), [b] – experimentally proven, [c] – transcription factor. HSP – heat-shock protein, LSU1 - low sulfur protein 1, TCP14 – TB1-CYC-PCF domain transcription factor 14. Screening of gene expression databases has been performed in September 2014. Predicted subcellular localizations are shown in order of decreasing probability score.

Chapter 2

Recombinant F-box-Nictaba protein interacts with *N*-acetyllactosamines, galactosylated structures and arabinan oligosaccharides

Part of this chapter has been published as:

Stefanowicz K, Lannoo N, Proost P, Van Damme EJM (2012). Arabidopsis F-box protein containing a Nictaba-related lectin domain interacts with *N*-acetyllactosamine structures. FEBS Open Bio 2, 151–158.

2.1 Abstract

The *Arabidopsis thaliana* genome contains a small group of bipartite F-box proteins, consisting of an N-terminal F-box domain and a C-terminal domain sharing sequence similarity with Nictaba, the jasmonate-induced glycan-binding protein (lectin) from tobacco. Based on the high sequence similarity between the C-terminal domain of these proteins and Nictaba the hypothesis was put forward that the so-called F-box-Nictaba proteins possess carbohydrate-binding activity and accordingly can be considered functional homologs of the mammalian sugar-binding F-box or Fbs proteins which are involved in proteasomal degradation of glycoproteins. To obtain experimental evidence for the carbohydrate-binding activity and specificity of the *A. thaliana* F-box-Nictaba proteins both the complete F-box-Nictaba sequence of one selected Arabidopsis F-box protein (*in casu* At2g02360) as well as the Nictaba-like domain only were expressed in *Pichia pastoris* and analyzed by affinity chromatography, agglutination assays and glycan microarray binding assays. Both proteins reacted similarly and screening of two different types of glycan arrays revealed that they recognize *N*- and *O*-glycans containing *N*-acetyllactosamine (Galβ1-3GlcNAc and Galβ1-4GlcNAc), poly-*N*-acetyllactosamine ([Galβ1-4GlcNAc]$_n$), Lewis A (Galβ1-3(Fucα1-4)GlcNAc), Lewis X (Galβ1-4(Fucα1-3)GlcNAc, Lewis Y (Fucα1-2Galβ1-4(Fucα1-3)GlcNAc) and blood type B (Galα1-3(Fucα1-2)Galβ1-3GlcNAc) motifs as well as β1-4-linked galactose oligomers and, with lower affinity, feruloylated α1-5-L-arabinobiose/triose glycans. These results demonstrated that the C-terminal Nictaba-like domain provides the F-box-protein with a carbohydrate-binding activity which is preferentially directed against glycan structures with a terminal galactose residue. Based on these findings one can reasonably conclude that at least the *A. thaliana* F-box-Nictaba protein encoded by *At2g02360* can act as a carbohydrate-binding protein. The results from the glycan array assays revealed differences in sugar-binding specificity between the F-box protein and Nictaba, indicating that the same carbohydrate-binding motif can accommodate unrelated oligosaccharides.

2.2 Introduction

The discovery of Nictaba, a jasmonate-induced lectin in tobacco (*N. tabacum*) leaves, made an important contribution to plant glycobiology during the last decade (Chen *et al.,* 2002; Van Damme *et al.,* 2004). For the first time evidence was presented that application of one plant hormone induced the expression of a specific lectin in plants. In addition, the identification of Nictaba as a cytoplasmic/nuclear lectin preferentially interacting with GlcNAc oligomers put the search for the physiological role of plant lectins in a new perspective. Subsequent *in silico* analyses revealed that the 165 AA residue subunit of the Nictaba homodimer represents a structural (carbohydrate-binding) motif that might well be ubiquitous in terrestrial plants.

True orthologs/homologs consisting of only Nictaba domain(s) are not widespread but numerous chimera proteins could be identified which possess a Nictaba domain fused to unrelated N- and C-terminal domains. Moreover, *in silico* analyses revealed that all currently sequenced plant genomes

contain genes encoding proteins with a Nictaba-like domain either or not fused to an unrelated domain (Van Damme *et al.,* 2008). For some of the expressed proteins, evidence could be presented that they possess lectin activity owing to their Nictaba domain. For example, the well documented family of phloem lectins from Cucurbitaceae, also known as the PP2 proteins (Read and Northcote, 1983; Sabnis and Hart, 1978) groups proteins with an active GlcNAc-binding Nictaba-like domain linked to an undefined N-terminal domain and a short cysteine-rich C-terminal peptide. The PP2-A1 protein from Arabidopsis is composed of an N-terminal domain followed by a Nictaba domain and has been shown to exhibit a carbohydrate-binding specificity similar to Nictaba (Beneteau *et al.,* 2010).

A comprehensive analysis of the Arabidopsis genome/transcriptome revealed the occurrence of chimera proteins in which a Nictaba-like domain is C-terminally fused to an F-box domain (Dinant *et al.,* 2003; Gagne *et al.,* 2002; Lannoo *et al.,* 2008). F-box domains are conserved, structural motifs of 40 – 50 AA residues commonly used in F-box proteins as a protein-protein interaction domain involved in the direct binding of F-box proteins to the protein Skp1 (Bai *et al.,* 1996). Complexes consisting of Skp, Cullin and an F-box protein (SCF complexes) play a role in Ub labeling of proteins destined for proteasomal degradation (Petroski and Deshaies, 2005a). Within F-box proteins, F-box domains are mostly used in concert with other (C-terminal) protein-protein interaction domains such as LRR or WD repeats. Based on this bipartite structure, F-box proteins can assemble into functional SCF complexes and recruit proteins that are destined for degradation to the complex for Ub labeling (Lechner *et al.,* 2006).

Although these F-box-Nictaba like sequences were first identified in Arabidopsis, homologs occur in all plant genomes sequenced thus far. Moreover, most plant transcriptomes comprise sequences encoding F-box-Nictaba chimers indicating that this group of proteins is widely expressed in plants (Lannoo *et al.,* 2008). Though not conclusive the apparently ubiquitous occurrence and expression of the genes indicate that the F-box-Nictaba proteins might fulfill a universal role in plants. Due to the presence of the F-box domain it is tempting to hypothesize that the F-box-Nictaba proteins are somehow involved in a proteasomal protein degradation process. This hypothesis follows the concept that the F-box-Nictaba proteins can be considered functional homologs of the mammalian Fbs proteins. These mammalian Fbs proteins also consist of an N-terminal F-box domain and a C-terminal carbohydrate-binding domain (unrelated to Nictaba), and are known to play a role in cytosolic proteasomal degradation of glycoproteins (Yoshida and Tanaka, 2010). Interestingly, the F-box-Nictaba protein under study, has been shown to interact with Skp1 homologs expressed in Arabidopsis (Arabidopsis Interactome Mapping Consortium, 2011; Takahashi *et al.,* 2004).

To test the hypothesis of being functional homologs of the mammalian Fbs proteins the presumed carbohydrate-binding activity of the Nictaba domain of the plant F-box-lectin needs to be supported by experimental evidence. Sequence alignments indicated that the deduced AA sequences of the Nictaba domain of *A. thaliana* F-box-Nictaba proteins share approximately 40% overall sequence similarity with Nictaba itself (Lannoo *et al.,* 2008). However, since this cannot guarantee that the carbohydrate-binding activity and specificity are conserved the lectin activity of the Arabidopsis F-box-Nictaba protein encoded by At2g02360 was investigated in detail. The *At2g02360* sequence was

selected because it shares the highest sequence similarity (64%) to Nictaba. To corroborate the carbohydrate-binding properties of this F-box-Nictaba protein the recombinant full length protein as well as the composing Nictaba domain were produced in *P. pastoris* and subsequently the recombinant proteins were purified and characterized. Here we show that both the entire F-box-Nictaba protein and its Nictaba domain possess carbohydrate-binding activity and exhibit virtually the same specificity. Studies using CFG glycan arrays revealed that both proteins preferentially bind *N*- and *O*-glycans containing *N*-acetyllactosamine (LacNAc) (Galβ1-3GlcNAc and Galβ1-4GlcNAc), poly-LacNAc ($[Galβ1\text{-}4GlcNAc]_n$), Lewis A (Galβ1-3(Fucα1-4)GlcNAc), Lewis X (Galβ1-4(Fucα1-3)GlcNAc, Lewis Y (Fucα1-2Galβ1-4(Fucα1-3)GlcNAc) and blood type B (Galα1-3(Fucα1-2)Galβ1-3GlcNAc) motifs. Furthermore, screening of the plant glycan microarrays revealed interaction with β1-4-linked Gal oligomers and, to a lesser extent, with feruloylated α1-5-L-arabinobiose/triose glycans. Collectively, these data suggest that F-box-Nictaba exhibits specificity towards glycan structures containing a terminal Gal residue. This specificity differs from the preferential binding of the tobacco lectin to high-Man *N*-glycans (Lannoo *et al.,* 2006b; Schouppe *et al.,* 2010) which illustrates once more that the same structural motif can accommodate different oligosaccharides depending on the atomic structure of the lectin binding site(s).

2.3 Materials and methods

2.3.1 Cloning of expression vectors for Arabidopsis F-box-Nictaba and its Nictaba domain for recombinant expression in *P. pastoris*

Cloning and heterologous expression of recombinant proteins were performed using the EasySelect™ *Pichia* Expression Kit from Invitrogen (Invitrogen, Carlsbad, CA, USA). The full-length cDNA template for At2g02360 (BX820545) was ordered from INRA (Institut National de la Recherche Agronomique), Centre de Toulouse, Unité de Recherche 1258-CNRGV (Centre National de Ressources Génomiques Végétales) (Castanet-Tolosan Cedex, France). The full-length F-box-Nictaba cDNA sequence was amplified by PCR using the forward primer evd553 and a reverse primer evd554 (supplementary Table A2.1) under the following PCR conditions: 2 min denaturation at 95˚C, 30 cycles of 15 s - 95˚C, 30 s - 60˚C, 1 min 20 s - 72˚C, additional 5 min elongation at 72˚C. Amplification of the Nictaba domain alone was achieved with forward primer evd360 and reverse primer evd359 (supplementary Table A2.1) using the following PCR conditions: 2 min denaturation at 94˚C, 25 cycles of 15 s - 94˚C, 30 s - 50˚C, 1 min - 72˚C, additional 5 min elongation at 72˚C. The amplified sequences were double digested with the restriction enzymes *Pml*I and *Sac*II (Fermentas, St. Leon-Rot, Germany) and the resulting fragment was cloned in the shuttle vector pPICZαA. After transformation into *E. coli* Top10F cells using heat shock transformation *E. coli* transformants were selected on LB agar plates containing zeocin (100 µg/ml) and checked by colony PCR using 5' and 3'- AOX1 specific primers (supplementary Table A2.1). PCR conditions were as follows: 12 min at 94˚C, 30 cycles of 15 s

- 94˚C, 30 s - 48˚C, 1 min - 72˚C, additional 5 min elongation at 72˚C. Plasmids from transformed *E. coli* colonies were purified using the E.Z.N.A. Plasmid Mini kit I (Omega Bio-Tek, Norcross, GA, USA). Afterwards, the sequences of the fusion constructs were verified by DNA sequencing (LGC Genomics, Berlin, Germany).

2.3.2 Transformation of *P. pastoris* and expression analysis

The plasmid DNA from selected *E. coli* cells was purified using the NucleoBond® Xtra Midi kit (Macherey-Nagel, Düren, Germany) and linearized using the restriction enzyme *Pme*I (plasmid construct for full-length F-box-Nictaba) or *Sac*I (plasmid construct for Nictaba domain) (Fermentas) with overnight incubation at 37˚C. Subsequently, *P. pastoris* competent cells were transformed with the linearized expression vector using electroporation (Bio-Rad, Hercules, CA, USA) with the following pulse settings: 25 µF, 1.5 kV and 125 Ω. Transformants were selected on YPDS plates (1% yeast extract, 2% peptone, 2% dextrose, 1M sorbitol, 2% agar) containing 300 µg/ml zeocin.

2.3.3 Optimization of recombinant protein expression in *P. pastoris*

For expression analysis, several colonies were inoculated in 5 ml BMGY medium containing 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base with ammonium sulfate and without AAs, $4x10^{-5}$% biotin, 100 mM potassium phosphate (pH 6.0), 1% glycerol and 100 µg/ml zeocin. Recombinant protein expression was optimized by testing different temperatures of culture incubation and different concentrations of methanol were used to induce recombinant protein expression. Cultures were grown at 22˚C or 30˚C in a shaker set at 220 rpm. After 24h of incubation *Pichia* cells were washed twice with sterilized water and transferred to 10 ml BMMY medium (BMGY medium containing 1% or 2% of methanol instead of 1% of glycerol). Cultures were grown for 4 days under the same conditions as before. Induction of recombinant protein expression was achieved by adding methanol twice a day (once in the morning and once in the evening) to a final concentration of 1% or 2%. Proteins in the culture medium were precipitated with trichloroacetic acid (10% final concentration) and analyzed by SDS-PAGE and Western blotting.

2.3.4 Large scale production of recombinant proteins

For expression of the recombinant proteins in large cultures, transformed *P. pastoris* colonies were inoculated into 10 ml BMGY medium and grown for 24h at 22˚C in a shaker at 220 rpm. Afterwards, cultures were transferred to 50 ml BMGY in 250 ml Erlenmeyer flasks and allowed to grow until the culture reached an optical density between 2 and 6 at 600 nm. Cells were then washed twice with sterilized water and resuspended in 250 ml of BMMY medium in 1L Erlenmeyer flasks. Cultures were

grown for 3 days under the same conditions as before. Induction of recombinant protein expression was achieved by adding methanol twice a day until a final concentration of 1%. After 3 days of methanol induction, cultures were centrifuged for 10 min at 3,000 g and the culture medium was collected. Proteins from the culture medium were precipitated using ammonium sulfate at a final concentration of 80%. The culture medium with precipitated proteins was stored at 4˚C until use.

2.3.5 Protein purification using column chromatography

Purification of recombinant proteins was achieved by 5 chromatographic steps. The culture medium with precipitated proteins was centrifuged at 17,000 g for 25 min and the resulting pellet was resuspended in phosphate buffered saline. After adjusting the ammonium sulfate concentration to 1 M and setting the pH to 7, the protein solution was loaded on a phenyl Sepharose column (GE Healthcare, Uppsala, Sweden) equilibrated with 1 M ammonium sulfate (pH 7). The column was washed with 1 M ammonium sulfate (pH 7) and eluted with 20 mM 1,3- diaminopropane. This eluted protein fraction was then loaded on a Q Fast Flow column equilibrated with 20 mM 1,3-diaminopropane. After washing with equilibration buffer bound proteins were eluted using 0.5 M NaCl/100 mM Tris-HCl (pH 8.7) buffer. After adding imidazole to a final concentration of 25 mM, the eluate from the Q Fast Flow column was applied on a Ni-Sepharose column (GE Healthcare) equilibrated with 0.5 M NaCl /25mM imidazole/100 mM Tris-HCl (pH 8,7) buffer to purify the His_6-tagged protein. The column was washed using the equilibration buffer and then stepwise elution of bound proteins was performed with 0.5 M NaCl/100 mM Tris-HCl (pH 8.7) buffer with increasing imidazole concentrations ranging from 50 to 250 mM imidazole. Fractions eluted from the Ni-Sepharose column were pooled and 1 M ammonium sulfate was added. After adjusting the solution to pH 7 the eluate was loaded on an ovomucoid-conjugated Sepharose 4B column equilibrated with 1 M ammonium sulfate (pH 7.0). After washing the ovomucoid-conjugated column with 1 M ammonium sulfate (pH 7.0), the bound proteins were eluted using 20 mM 1,3-diaminopropane. Finally this protein fraction was concentrated on a small Q Fast Flow column. The purified proteins were eluted into 0.5 M NaCl/20 mM 1,3-diaminopropane (pH 8.7) buffer. The purity of the protein samples after each purification step was verified by SDS-PAGE and Western blot analysis.

2.3.6 SDS-PAGE and Western blot

SDS-PAGE was performed using 15% polyacrylamide gels under reducing conditions (Laemmli, 1970). Proteins were visualized by gel staining with Coomassie Brilliant Blue R-250. For Western blot analysis, samples separated by SDS-PAGE were electrotransferred to 0.45 µm polyvinylidene fluoride membranes (Biotrace™ PVDF, PALL, Gelman Laboratory, Ann Arbor, MI, USA). Membranes were blocked with 5% (w⁄v) milk powder in Tris-buffered saline (TBS: 150 mM NaCl, 10 mM Tris, 0.1% (v⁄v) Triton X-100, pH 7.6). Afterwards blots were incubated for 1 h with a mouse monoclonal anti-His_6 (C-

terminal; Invitrogen) or a mouse monoclonal anti-c-Myc antibody (Invitrogen) diluted 1⁄5,000 in TBS, washed 3 times in TBS and finally incubated with the 1⁄1,000 diluted rabbit anti-mouse IgG secondary antibody labeled with horseradish peroxidase (Dako Cytomation, Glostrup, Denmark). Immundetection was performed using a colorimetric assay with 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St Louis, MO, USA) as a substrate. All washes and incubations were conducted at room temperature on a platform with gentle shaking.

2.3.7 Agglutination assays

Agglutination assays to check for lectin activity of purified recombinant protein fractions were performed as described by Al Atalah *et al.* (2011). 10 µl of purified protein fractions (0.5 mg/ml) were mixed with 10 µl 1 M ammonium sulfate and 30 µl of a 2% solution (in 1x PBS) of trypsin-treated rabbit red blood cells (Bio-Mérieux, Marcy l'Etoile, France). Agglutination was observed after 20 min.

2.3.8 N-terminal sequence analysis

Samples of purified recombinant F-box-Nictaba protein and its Nictaba domain alone were analyzed by SDS-PAGE, electroblotted onto a BioTrace™ polyvinylidene fluoride membrane (Gelman Laboratory) and stained with a 1:1 mix of Coomassie Brilliant Blue and methanol. Protein bands were excised from the membrane and the N-terminal sequence was determined by Edman degradation on a capillary Procise 491cLC protein sequencer without alkylation of cysteines (Applied Biosystems).

2.3.9 Analysis of recombinant proteins on CFG glycan microarrays

The printed microarrays containing glycans covalently-coupled to glass slides and validated using glycan-binding molecules (http://glycomics.scripps.edu/coreD/DGlycanArrayReagent.pdf) are described by Blixt *et al.* (2004). Printed array version 5.0 used for the analyses is reported here (http://www.functionalglycomics.org/static/consortium/resources/resourcecoreh8.shtml). Purified proteins were labeled using the Alexa Fluor®488 Protein Labeling Kit (Invitrogen) following the manufacturer's instructions. The labeled proteins diluted in binding buffer (TBS containing 10 mM $CaCl_2$, 10 mM $MgCl_2$, 1% (w⁄v) BSA, 0.05% (v/v) Tween 20) to 200 µg/ml were applied to separate microarray slides and incubated under a cover slip for 1 h in a dark, humidified chamber at room temperature. Then, the cover slips were gently removed in a solution of TBS containing 0.05% (v/v) Tween 20 and washed by dipping the slides four times in successive washes of TBS containing 0.05% (v/v) Tween 20, TBS, and deionized water. After the last wash, the slides were spun in a slide centrifuge for approximately 15 s to dry. Immediately after that, the slides were scanned in a PerkinElmer ProScanArray MicroArray Scanner using an excitation wavelength of 488 nm and

ImaGene software (BioDiscovery Inc., El Segundo, CA USA) to quantify the fluorescence. The data were reported as average Relative Fluorescence Units (RFU) of four replicate values after removal of the high and low values of the six replicates of each glycan presented on the array. The complete primary data set for each protein is available on the website of the Consortium for Functional Glycomics (http://www.functionalglycomics.org).

2.3.10 Analysis of recombinant proteins on plant glycan microarrays

The plant glycan microarrays containing glycans printed on nitrocellulose membrane are described by Pedersen *et al.* (2012) and have been provided by Prof. WGT Willats (Department of Plant and Environmental Sciences, University of Copenhagen, Denmark). In short, glycan arrays were first incubated for 1 h in blocking solution consisting of 3% (w⁄v) BSA in 1x phosphate-buffered saline (1x PBS). Then, the arrays were probed for 2 h with 10 µg/ml of purified proteins dissolved in blocking solution. Afterwards, arrays were washed 3 times in 1x PBS and incubated for 1 h with a mouse monoclonal anti-His_6 antibody (Thermo Scientific) diluted 1⁄3,000 in blocking solution. Next, they were washed again and incubated for 1 h with a rabbit anti-mouse polyclonal secondary antibody coupled to alkaline phosphatase (Thermo Scientific) diluted 1⁄10,000 in blocking solution. Prior to detection, arrays were washed in 1x PBS and finally briefly rinsed with ultrapure MilliQ water. Immunodetection was performed using the chromogenic 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium substrate prepared by dissolving ½ of SIGMA*FAST®* BCIP/NBT tablet (Sigma-Aldrich) in 10 ml of ultrapure MilliQ water. The reaction was stopped by washing the arrays with ultrapure MilliQ water as soon as detection signals became visible. All washes and incubations were conducted at room temperature on a platform with gentle shaking. As negative control, a plant glycan array was probed with blocking solution.

Arrays were scanned with a flatbed scanner (Canoscan Lide 25, Canon, Diegem, Belgium) at 1200 dpi. Signal intensities were assessed via the ImageJ software package using the MicroArray Profile plugin (by Dougherty and Rasband; OptiNav, Inc.) and were corrected by subtracting the local background measured in the closest proximity of the array spots. The significance cut-off or signal intensity was calculated as the highest intensity value for blank sample score plus standard error of all blank intensities.

2.4 Results

2.4.1 Trp residues essential for glycan binding are conserved in the Arabidopsis F-box-Nictaba protein

Sequence alignments revealed that the Nictaba domain of the Arabidopsis F-box-Nictaba protein At2g02360 (AtPP2-B10) exhibits 52% and 64% sequence identity and similarity at the AA level, respectively, with the Nictaba sequence deduced from the cDNA encoding the *N. tabacum* leaf lectin (Chen *et al.,* 2002). According to a recently reported three-dimensional model for Nictaba (Schouppe *et al.,* 2010), an electronegatively charged region on the protein surface was predicted as the glycan-binding site. Mutational analyses of two tryptophan residues (Trp15 and Trp22) located in the N-terminal half of the Nictaba domain revealed that these two residues are essential for glycan binding activity of the tobacco lectin since replacement of these Trp residues by Leu completely abolished the interaction of the mutant protein with the glycan array (Schouppe *et al.,* 2010). Interestingly, sequence alignments of the Nictaba domain of At2g02360 and the tobacco lectin itself (Fig. 2.1) revealed that these Trp residues (Trp118 and Trp125 in At2g02360) are highly conserved in the F-box protein suggesting potential lectin activity for the Arabidopsis F-box-Nictaba protein with a similar sugar specificity as the one reported for the lectin from tobacco.

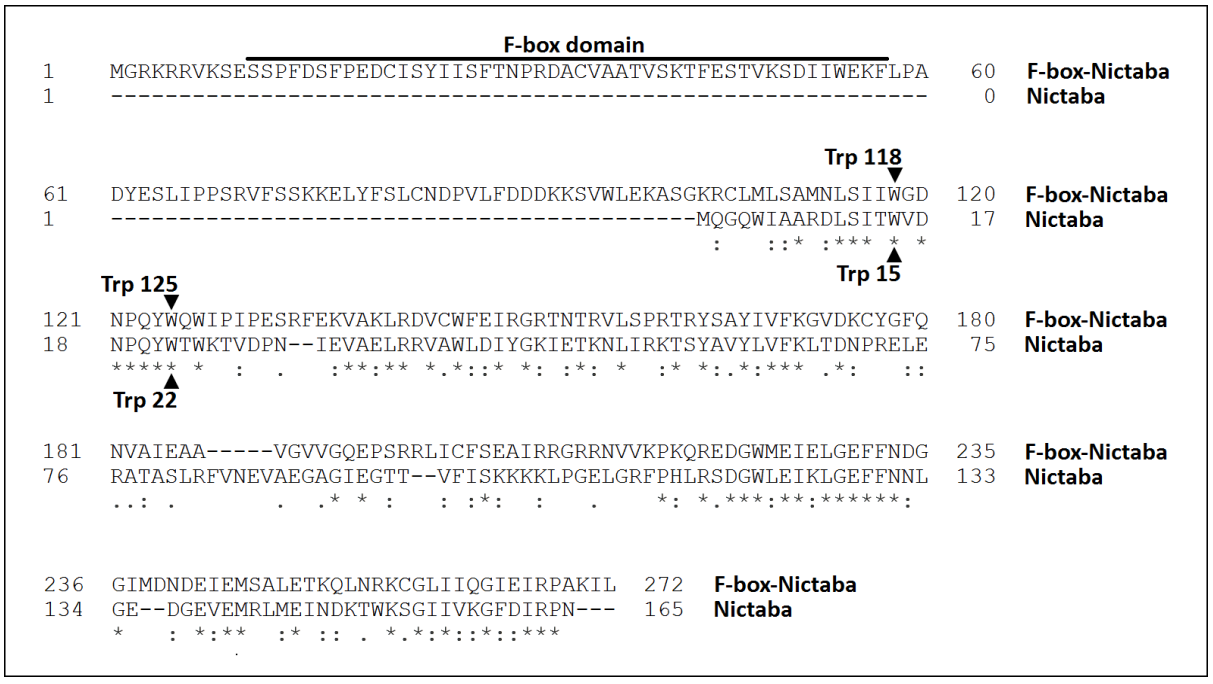


Fig. 2.1 Sequence alignment of Nictaba and the Arabidopsis F-box-Nictaba protein.

2.4.2 Recombinant protein expression in *P. pastoris*

To corroborate the glycan-binding activity and specificity of At2g02360 both the holoprotein (AA residues 1-272) and the composing Nictaba domain (AA residues 95-272) were expressed in the yeast *P. pastoris.* Sequences corresponding to the full-length protein and the Nictaba domain, respectively, were amplified by PCR and cloned in the pPICZαA vector, which was subsequently transferred into *P. pastoris* strain KM71H. Expression of recombinant F-box-Nictaba as well as the Nictaba domain was tested in small scale experiments. For each construct, several cultures were incubated at 30°C and recombinant protein expression was induced using 2% methanol according to Al Atalah *et al.* (2011). In order to detect a signal of recombinantly produced proteins it was necessary to extract protein from 10 ml of medium. As shown in Fig. 2.2A, a few cultures transformed with the F-box-Nictaba construct were producing His-tagged protein. Nevertheless, instead of the expected 35 kDa protein, two distinct bands could be detected at approximately 55 kDa. The specificity of the signal was confirmed by a Western blot assay using an anti-c-Myc antibody on the protein extracts from the same *Pichia* culture (results not shown). All tested cultures for Nictaba-like domain expression were producing His-tagged protein with different yields (Fig. 2.2B). Instead of one band of approximately 24 kDa, however, two separate signals were detectable around 26 kDa.
The appearance of recombinant F-box-Nictaba as a protein of much higher molecular weight (MW) than calculated based on its AA sequence, could suggest some post-translational modification. This modification could be due to the fact that the protein is synthesized on the ER and will be secreted by *Pichia* into the medium. One of the most common and highly complex post-translational modifications performed by *P. pastoris* on secreted proteins is glycosylation (Macauley-Patrick *et al.,* 2005). Many proteins recombinantly expressed in *P. pastoris* as secreted proteins have been shown to be extensively *N*- and *O*-glycosylated. In fact, several reports describe a recombinant protein size increase due to hypermannosylation by over 20 kDa (Heimo *et al.,* 1997; Teh *et al.,* 2011; Trimble *et al.,* 2004). Alternatively, it could be speculated that F-box-Nictaba forms dimers by disulphide bond formation during protein passage through the ER and Golgi before final secretion.

Due to very low yields of recombinant protein production, the conditions for growth and induction of *P. pastoris* were optimized to enhance recombinant protein expression. Normally, 30°C is recommended for optimal growth of *Pichia* and allows successful recombinant protein expression (*Pichia* Expression Kit, Invitrogen; Al Atalah *et al.,* 2011). However, it has been reported that cultures grown at lower temperatures might be more favorable in view of recombinant protein yield by limiting extracellular proteolysis and minimizing aggregate formation (Dragosits *et al.,* 2009; Huang *et al.,* 2011; Jahic *et al.,* 2003; Li *et al.,* 2001; Shi *et al.,* 2003). Thus, our first approach to optimize protein yield included growth and induction of yeast cultures at 22°C. First trials were performed using the *Pichia* colony showing the highest expression level for the Nictaba-like domain at 30°C. As shown in Fig. 2.2C (upper part), the Nictaba domain was well detectable (altogether with some protein degradation products) in the crude protein extract originating from 1 ml of medium from the culture grown at 22°C. In contrast, when the culture was grown at 30°C the protein band for the

Nictaba domain was barely visible. There were no clear differences in recombinant protein expression levels when induction was performed with 1% vs. 2% methanol (Fig. 2.2C. lower panel).
Based on this finding, new experiments were performed with several *Pichia* colonies with incubation of cultures at 22°C and induction of recombinant protein expression using 1% methanol. Fig. 2.2D and E show the resulting protein expression with these new culture settings. Fig. 2.2D demonstrates that recombinant F-box-Nictaba was present in most of the protein extracts prepared from 1 ml culture medium of transformed yeast cells. Interestingly, the protein was detectable as a single polypeptide and its size corresponded to the calculated 35 kDa, in contrast to the results obtained for cultures grown at 30°C and treatment with 2% methanol (Fig. 2.2A). Similarly, all tested cultures for Nictaba domain expression were efficiently producing the recombinant protein of roughly correct size, although two separate His_6-tag-labeled polypeptides were still detectable (Fig. 2.2E). Some bands were also visible far below 26 kDa, presumably constituting degradation products. Thus, the lower incubation temperature of *Pichia* cultures significantly enhanced recombinant protein yield for both the full-length F-box-Nictaba and its Nictaba domain only. In general, recombinant protein yield per ml of culture appeared higher for the Nictaba-like domain than for the F-box-Nictaba, regardless of growth and induction conditions.
The *Pichia* strains with the highest expression levels were selected and used for the production of the recombinant proteins.

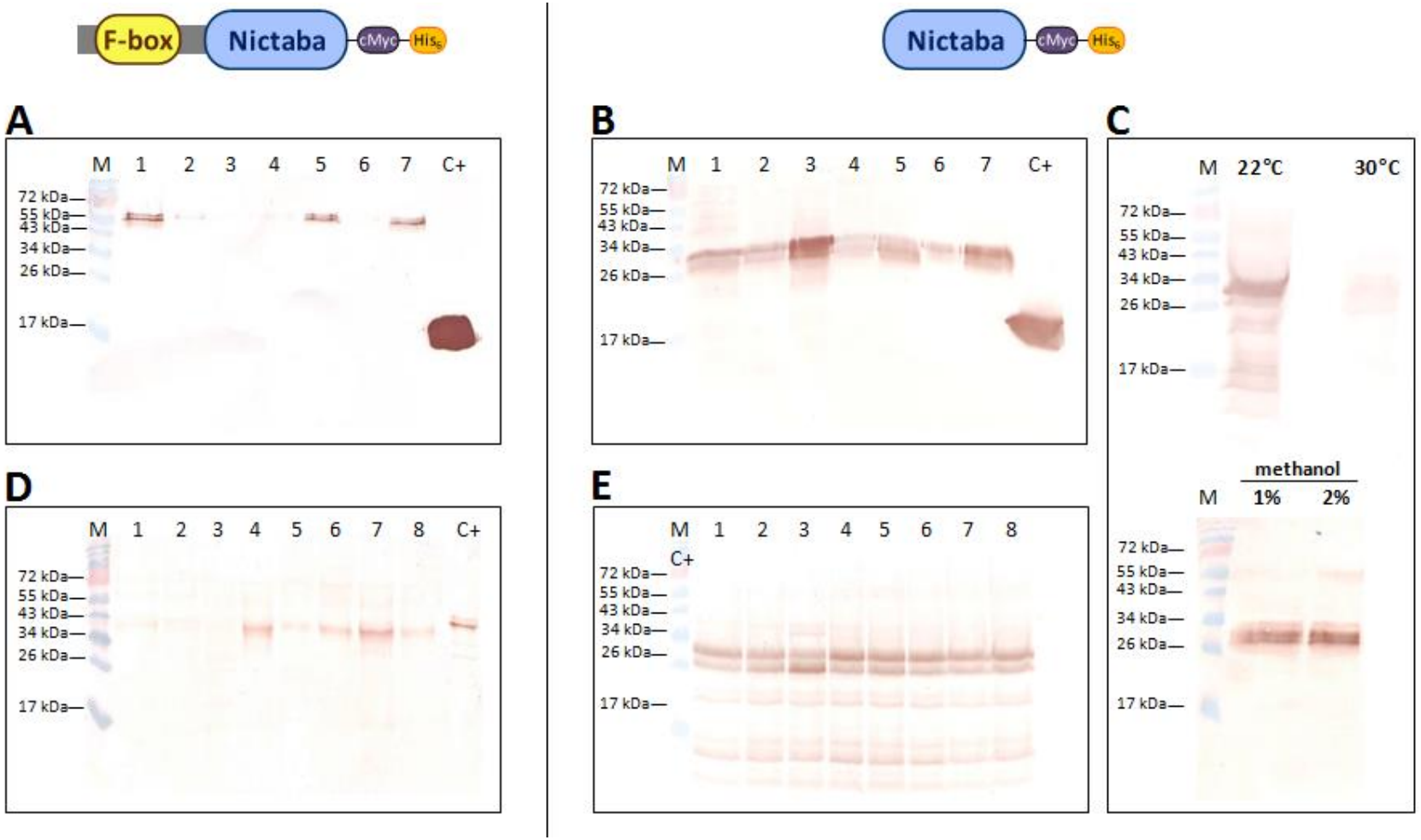


Fig. 2.2 Western blot pictures of crude protein fractions from culture medium of different *P. pastoris* transformants containing recombinant F-box-Nictaba protein (A,D) or the Nictaba domain from the F-box-Nictaba protein (B, C, E). A and B, Protein fractions originating from 10 ml of culture medium of yeast cells grown at 30°C in BMMY with 2% MeOH. C, Upper panel: Protein fractions originating from 1 ml of culture medium of yeast colony no 3 (from panel B) grown at 22°C or 30°C in BMMY with 2% MeOH; Lower panel: fractions originating from 1 ml of culture medium of yeast colony no 3 (from panel B) grown at 22°C in BMMY with either 1% or 2% MeOH. D and E, Protein fractions originating from 1 ml of culture medium of yeast cells grown at 22°C in BMMY with 1% MeOH. M: protein marker; C+: positive control (His_6-tagged protein).

2.4.3 Large scale production of recombinant proteins and protein purification

SDS-PAGE analysis followed by Western blot analysis of crude protein extracts confirmed the successful expression of the His-tagged full-length protein as well as of the Nictaba domain in *Pichia* large scale fed batch cultures (Figs. 2.3A-B). Both recombinant proteins were purified using a combination of ion exchange chromatography, metal affinity chromatography on a Ni-Sepharose column and affinity chromatography on an ovomucoid-Sepharose 4B matrix. SDS-PAGE and Western blot analysis confirmed the purity of the protein preparations (Figs. 2.3C-D). Yields amounted to approximately 250 µg and 500 µg per liter culture for the recombinant full-length F-box protein and recombinant Nictaba-like domain, respectively. The molecular mass of the recombinant F-box-Nictaba was estimated 37-38 kDa, which is in good agreement with the calculated molecular mass of 34.9 kDa of the recombinant protein (including a c-Myc epitope and a His_6-tag). Unlike the recombinant F-box-Nictaba, which yielded a single polypeptide, the purified recombinant Nictaba domain yielded two major polypeptides of approximately 26 and 24 kDa, respectively. N-terminal AA sequencing of the recombinant Nictaba domain yielded the sequence EAEAEFSVXLEEA with 85% sequence identity to the N-terminus of the recombinant protein expressed in *Pichia*, and revealed that the higher molecular mass of the 26 kDa polypeptide is due to an incomplete removal of the N-terminal secretion sequence needed to direct the expressed proteins into the culture medium. The protein patterns shown in Figures 2.3A-D suggest partial degradation of the recombinant proteins. Nevertheless, these polypeptides could be detected using specific anti-His antibodies after purification on the ovomucoid column. Taken into account that the smaller protein polypeptides were also bound to the ovomucoid column these polypeptides also exhibit carbohydrate-binding activity.

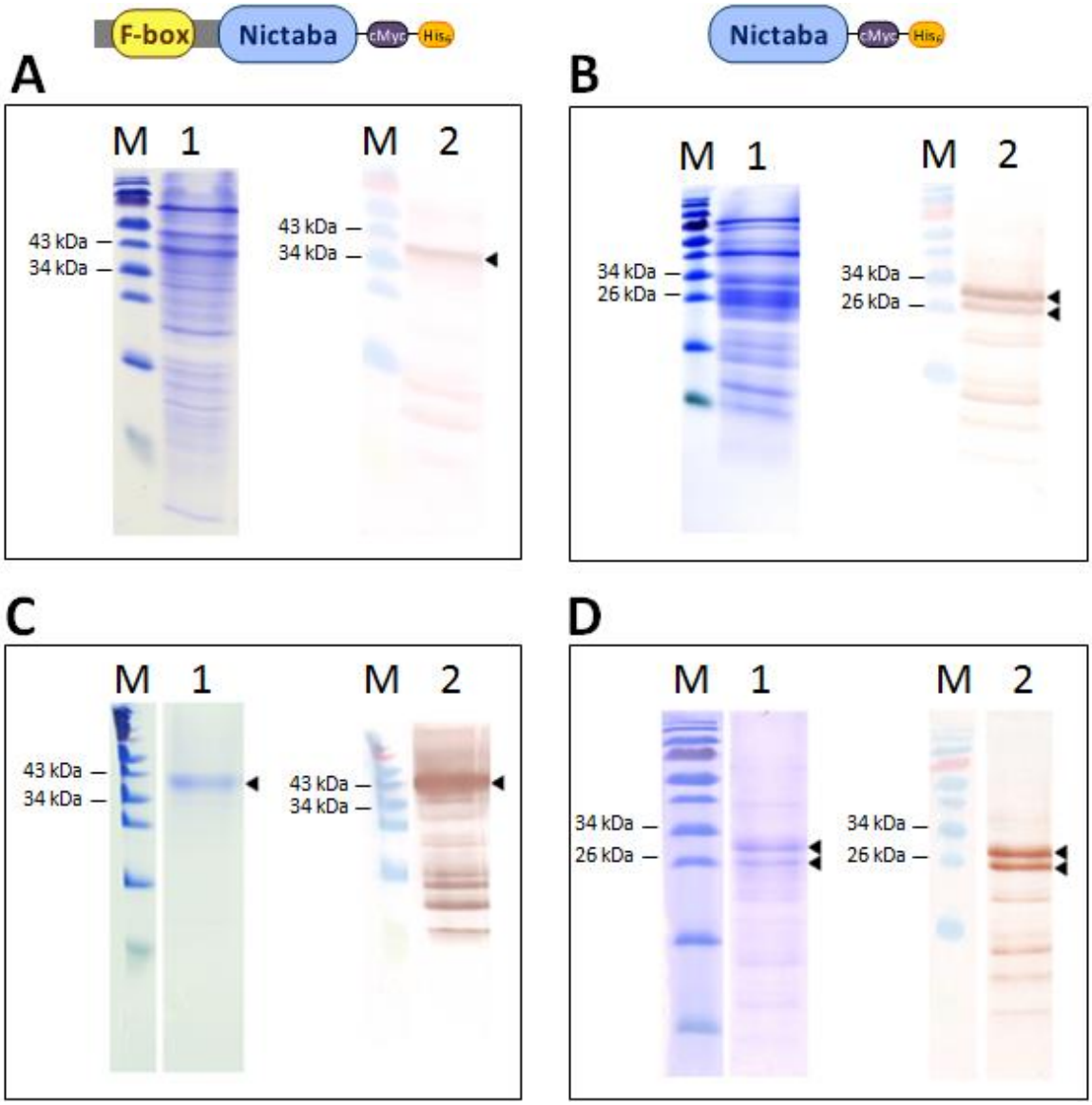


Fig. 2.3 SDS-PAGE and Western blot pictures of unpurified and purified protein fractions**.** A, Crude protein extract from *P. pastoris* culture medium containing the recombinant F-box-Nictaba protein (50 µg/lane). B, Crude protein extract from *P. pastoris* culture medium containing the recombinant Nictaba domain from At2g02360 (50 µg/lane). C, Purified recombinant F-box-Nictaba protein (2 µg/lane). D, Purified recombinant Nictaba domain from At2g02360 (2 µg/lane). M: protein marker; lane 1 for each panel: SDS-PAGE image; lane 2 for each panel: Western blot image.

2.4.4 Lectin activity of recombinant F-box-Nictaba

The fact that both recombinant proteins could be isolated by affinity chromatography on immobilized ovomucoid, a highly glycosylated protein carrying high-Man N-glycans, already indicated that they possess carbohydrate-binding activity. Furthermore agglutination assays with the purified proteins also yielded a positive reaction in an agglutination test with rabbit erythrocytes (Results not shown).

Nevertheless, in order to study the F-box-Nictaba lectin specificity more sophisticated methodology was used. Glycan microarray technology, comprising a wide range of diverse carbohydrates

immobilized on a solid support, has become a powerful tool for exploring carbohydrate-mediated interactions in a high-throughput mode (Park *et al.,* 2013). Glycan array screening has been extensively used in glycomics for a fast assessment of carbohydrate-binding properties of antibodies and lectins and quantitative analysis of protein-carbohydrate interactions, as well as in biological and biomedical research for detection of cells and pathogens or identification of anti-glycan antibodies for clinical diagnostics (Katrlík *et al.,* 2011; Liang and Wu, 2009; Park *et al.,* 2008). In this work, two different types of glycan microarrays were used to identify the carbohydrate-binding specificity of both recombinant proteins. The glycan-binding activity of purified F-box-Nictaba and its Nictaba-like domain produced in *P. pastoris* as well as of Nictaba from tobacco recombinantly expressed and purified before (Schouppe *et al.,* 2010) was determined using the printed glycan array_v5.0 available from the Consortium for Functional Glycomics (USA) containing over 600 purified and synthesized glycan structures (Blixt *et al.,* 2004; overview of glycan structures on glycan array_v5.0 are available at http://www.functionalglycomics.org/static/consortium/resources/resourcecoreh16.shtml). Furthermore, the lectin activity of purified F-box-Nictaba and its Nictaba-like domain produced in *P. pastoris* and the recombinant Nictaba protein from tobacco produced and purified as described by Vandenborre *et al.* (2008) was tested on plant glycan microarrays (Department of Plant and Environmental Sciences, University of Copenhagen, Denmark) (Pedersen *et al.,* 2012). These glycan arrays are nitrocellulose arrays with 126 different carbohydrate structures spotted on them. The glycans are predominantly plant-specific structures including both oligo- as well as polysaccharides. All saccharide structures are printed in duplicates at three different concentrations: 1, 0.2 and 0.04 mg/ml as well as 2, 0.4 and 0.08 mg/ml for poly- and oligosaccharides, respectively. An overview of all carbohydrates and their location on the array is presented in supplementary Tables A2.2 and A2.3.

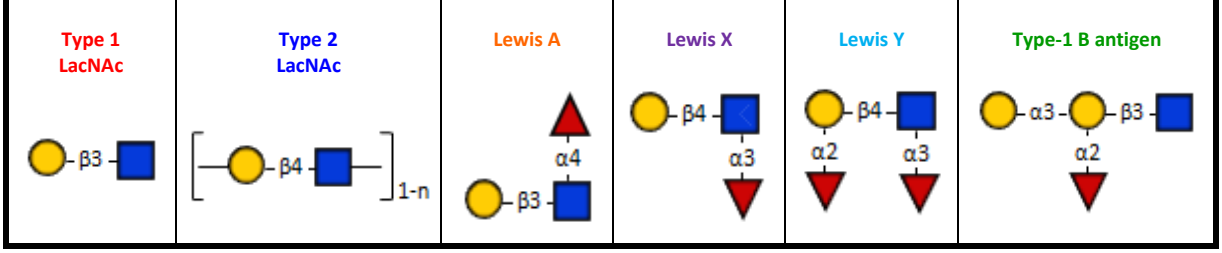
2.4.4.1 F-box-Nictaba interacts with *N*-acetyllactosamine structures

Table 2.1 summarizes the top 30 glycan structures most significantly (%CV < 40%) bound by the Nictaba domain of Arabidopsis F-box-Nictaba on the printed CFG glycan array_v5.0. As shown in Table 2.1, the Nictaba domain as well as the full-length F-box-Nictaba protein showed interaction towards both *N*- and *O*-glycans containing type 1 and type 2 LacNAc (Galβ1-3GlcNAc and Galβ1-4GlcNAc) and poly-LacNAc type 2 ($[Galβ1\text{-}4GlcNAc]_n$) structures as well as Lewis A (Galβ1-3(Fucα1-4)GlcNAc), Lewis X (Galβ1-4(Fucα1-3)GlcNAc), Lewis Y (Fucα1-2Galβ1-4(Fucα1-3)GlcNAc) and blood type B (Galβ1-3(Fucα1-2)Galβ1-3GlcNAc) epitopes. It is apparent that the presence of the F-box domain does not hamper the glycan-binding capacity of the lectin domain since the glycan interaction profile of the full-length protein is very similar to the one obtained for the lectin domain. Therefore, the Nictaba domain present in the Arabidopsis At2g02360 protein can be considered a functional lectin domain which exhibits specificity for the Gal-GlcNAc sequence, both in a β1-3 and a β1-4 linkage.

Table 2.1 Overview of the top 30 glycan structures with highest reactivity on the glycan array for the Nictaba domain of F-box-Nictaba from *A. thaliana* tested at 200 µg/ml on glycan array version 5.0. The most recurrent structures are shown at the bottom and are coloured in the table as follows: type 1 LacNAc motifs – red, type 2 LacNAc motifs – dark blue, Lewis A structures – orange, Lewis X structures – violet, Lewis Y structures – blue and type-1 B antigen structures – green. Glycan structures for which the % RFU for Nictaba from tobacco are > 10 % are marked in gray. The core motif of *N*-glycans known to be bound by tobacco Nictaba with the highest affinity ($Man_3GlcNAc_2$; Gheysen, 2011) is highlighted in yellow.

[a] % RFU, Percentage relative fluorescence units: relative value of the signal intensity for a glycan, calculated as % ratio of RFU of this glycan to the RFU of the glycan which showed the highest interaction on the array with the particular protein. Glycans which showed the highest interaction on the array were: glycan # 115 for the Nictaba domain and F-box-Nictaba from *A.thaliana*; glycan # 473 for Nictaba from tobacco - see Table 2.2.

Glycan #	Glycan structure	% RFU[a]		
		Nictaba domain	F-box-Nictaba	Nictaba from tobacco
115	Galα1-3Galβ1-3GlcNAcβ-Sp0	100	100	0.12
34	**(3S)**Galβ1-4**(6S)**GlcNAcβ-Sp0	76	42	0.13
25	**(3S)**Galβ1-4Glcβ-Sp8	51	14	0.19
102	Galα1-3(Fucα1-2)Galβ1-3GlcNAcβ-Sp0	48	28	0.13
534	Fucα1-4(Galβ1-3)GlcNAcβ1-2 Manα-Sp0	47	25	0.15
330	Neu5,9Ac2α2-3Galβ1-3GlcNAcβ-Sp0	44	14	0.04
26	**(3S)**Galβ1-4**(6S)**Glcβ-Sp0	42	14	0.10
396	Galα1-3Galβ1-3(Fucα1-4)GlcNAcβ1-2Manα1-6(Galα1-3Galβ1-3(Fucα1-4)GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-Sp19	35	32	14
275	Neu5Acα2-6Galβ-Sp8	35	8.1	0.10
314	Manα1-6Manβ-Sp10	34	16	0.35
126	Galβ1-2Galβ-Sp8	33	9.2	0.10
366	Fucα1-4(Galβ1-3)GlcNAcβ1-2Manα1-6(Fucα1-4(Galβ1-3)GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-Sp22	32	11	13
23	**6S(3S)**Galβ1-4GlcNAcβ-Sp0	32	11	0.15
325	Galβ1-3GlcNAcβ1-2Manα1-6(Galβ1-3GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp19	30	9.3	83
29	**(3S)**Galβ1-3GalNAcα-Sp8	29	7.0	0.14
252	Neu5Acα2-3Galβ1-4**(6S)**GlcNAcβ-Sp8	29	14	2.4
395	Galα1-3Galβ1-3GlcNAcβ1-2Manα1-6(Galα1-3Galβ1-3GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-Sp19	27	16	6.5
420	Galβ1-4(Fucα1-3)GlcNAcβ1-2Manα1-6(Galβ1-4(Fucα1-3)GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-Sp22	27	9.8	13
72	Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ-Sp0	27	2.9	0.10
527	Neu5Acα2-3Galβ1-3GlcNAcβ1-2Manα-Sp0	27	5.8	3.4
156	Galβ1-4**(6S)**Glcβ-Sp0	26	7.6	0.27
92	GalNAcα1-3GalNAcβ-Sp8	26	7.5	0.25
69	Fucα1-2Galβ1-3GlcNAcβ-Sp8	25	7.4	0.09
24	**(3S)**Galβ1-4(Fucα1-3)**(6S)**Glc-Sp0	23	5.3	0.34
468	Galα1-3(Fucα1-2)Galβ1-3GalNAcα-Sp8	23	0.9	0.22
560	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2)Manα1-6(Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-3)Manα1-4GlcNAcβ1-4GlcNAc-Sp24	23	28	10
36	**(3S)**Galβ1-4GlcNAcβ-Sp0	22	14	0.11
110	Galα1-4(Galα1-3)Galβ1-4GlcNAcβ-Sp8	22	18	0.36
22	**6S(3S)**Galβ1-4**(6S)**GlcNAcβ-Sp0	22	14	0.10
273	Neu5Acα2-6Galβ1-4Glcβ-Sp0	22	1.5	0.15



Moreover, Table 2.1 shows that out of the top 30 glycans preferentially bound by the Nictaba domain of F-box-Nictaba, only 5 carbohydrate structures were efficiently recognized by Nictaba from tobacco (glycans marked in gray, showing % RFU > 10 % for Nictaba). These are the only glycans in the top 30 presented in Table 2.1 which, apart from LacNAc-related structures, comprise also the core $Man_3GlcNAc_2$ motif of *N*-glycans (highlighted in yellow) known to be bound by tobacco Nictaba with the highest affinity (Gheysen, 2011).

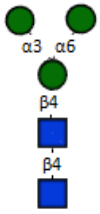
Previously, lectin from tobacco plants was shown to preferentially interact with GlcNAc oligomers and high-Man *N*-glycans when tested on the first generation arrays (v2.1, v3 and v4) (Lannoo *et al.,* 2006b; Schouppe *et al.,* 2010). A new screening using the latest available array v5 showed that Nictaba from tobacco also recognizes structures comprising LacNAc motifs (Table 2.2). However, in contrast to the Nictaba domain from the Arabidopsis F-box-Nictaba protein under study (Table 2.1), the tobacco lectin interacts with LacNAc-containing structures which are exclusively present as part of complex *N*-glycans containing the core $Man_3GlcNAc_2$ motif, but does not bind to simple lactosamine motifs. Table 2.2 also reveals that the full-length F-box Nictaba or its Nictaba domain can moderately interact with some of the LacNAc-containing complex *N*-glycans (at % RFU >10 %, but not exceeding 30 %) from the top 30 carbohydrate structures recognized by the tobacco lectin. Nevertheless, it is clearly demonstrated that in contrast to Nictaba, they cannot efficiently bind high-Man *N*-glycans and GlcNAc oligomers.

Table 2.2 Overview of the top 30 glycan structures with highest reactivity on the glycan array for Nictaba from tobacco ▶ tested at 200 µg/ml on glycan array version 5.0. The most recurrent structures are shown at the bottom of the table. The core motif of *N*-glycans known to be bound by tobacco Nictaba with highest affinity ($Man_3GlcNAc_2$; Gheysen, 2011) is highlighted in yellow. Glycan structures for which the % RFU for F-box-Nictaba from *A. thaliana* or for its Nictaba domain are > 10 % are marked in gray. Motifs preferentially bound on the glycan array by the Nictaba domain of F-box-Nictaba from *A. thaliana* (presented in Table 2.1) are coloured as follows: type 1 LacNAc motifs – red, type 2 LacNAc motifs – dark blue, Lewis A structures – orange, Lewis X structures – violet, Lewis Y structures – blue and type-1 B antigen structures – green.

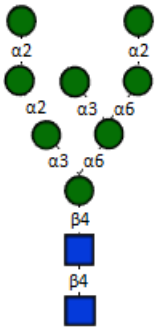
[a] % RFU, Percentage relative fluorescence units: relative value of the signal intensity for a glycan, calculated as % ratio of RFU of this glycan to the RFU of the glycan which showed the highest interaction on the array with the particular protein. Glycans which showed the highest interaction on the array were: glycan # 115 for the Nictaba domain and F-box-Nictaba from *A.thaliana* (see Table 2.1); glycan # 473 for Nictaba from tobacco.

Glycan #	Glycan structure	% RFU[a]		
		Nictaba domain	F-box-Nictaba	Nictaba from tobacco
473	Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-2Manα1-6(Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-Sp24	1.2	5.4	100
216	Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp12	2.5	6.1	99
325	Galβ1-3GlcNAcβ1-2Manα1-6(Galβ1-3GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp19	30	9.3	83
302	Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp12	7.2	31	79
212	Manα1-2Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp12	4.2	3.7	78
362	Fucα1-2Galβ1-4GlcNAcβ1-2Manα1-6(Fucα1-2Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp20	19	18	73
364	Galα1-3Galβ1-4GlcNAcβ1-2Manα1-6(Galα1-3Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp20	15	16	72
352	Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp12	3.6	6.1	71
51	Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp13	1.2	0.71	70
581	GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-6(GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-Sp19	1.1	1.9	68
372	Galα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-2Manα1-6(Galα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp20	15	8.5	68
53	GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp13	2.4	3.0	67
350	Galβ1-4GlcNAcβ1-2Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc-Sp12	2.4	1.2	67
545	Fucα1-2Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-6(Fucα1-2Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp24	3.1	19	67
486	Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ1-2)Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-Sp24	1.2	2.6	66
191	GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ1-Sp8	6.4	3.7	66
575	GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-6(GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-Sp24	2.2	3.8	66
328	Galβ1-4(Fucα1-3)GlcNAcβ1-2Manα1-6(Galβ1-4(Fucα1-3)GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp20	2.9	20	65
459	Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ1-2)Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp19	6.2	8.3	65
217	Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp12	1.9	19	65
550	GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-6(GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp25	18	2.2	63
577	GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-6(GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-Sp24	3.1	1.8	62
561	Galα1-3Galβ1-4GlcNAcβ1-2Manα1-6(Galα1-3Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-Sp24	1.8	21	62
211	Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp12	3.5	2.5	61
541	GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-6(GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp25	2.4	2.9	60
347	Manα1-6(Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-Sp12	5.7	5.6	59
485	Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-Sp19	1.2	0.87	59
582	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-Sp19	2.2	19	59
405	Galα1-4Galβ1-4GlcNAcβ1-2Manα1-6(Galα1-4Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp24	4.6	8.0	58
543	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp24	2.0	5.4	56

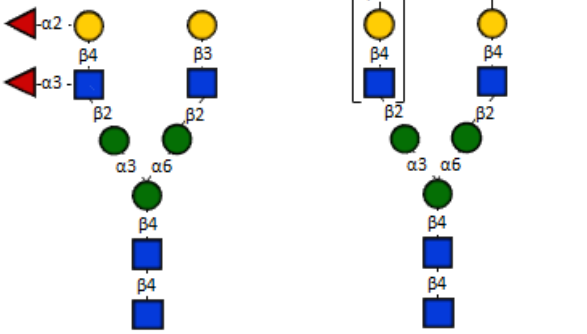
$Man_3GlcNAc_2$ core



High-Man type *N*-glycans



Complex type *N*-glycans



GlcNAc oligomers

2.4.4.2 F-box-Nictaba interacts with galactose oligomers and arabinan oligosaccharides

Probing the plant glycan arrays with the purified proteins of interest exhibiting carbohydrate-binding activity towards glycans present on the array results in a specific dot pattern as presented in Fig. 2.4A. Quantitative analysis of pixel intensities for all spots on the arrays after screening with F-box-Nictaba as well as with its Nictaba domain revealed that both proteins demonstrate very similar glycan-binding properties, although the signal intensities in general are lower for the complete F-box-Nictaba protein. In line with this result, a general observation was made that F-box-Nictaba appears to be much less stable in solution than its Nictaba domain. Also, it has been suggested that F-box proteins are regulated via an autoinhibitory mechanism and a non-occupied F-box domain impedes substrate binding, thereby ensuring that free F-box proteins do not compete with complete SCF complexes for access to targets (Chae et al., 2008; Deshaies, 1999). Fig. 2.4B and Fig. 2.5 present the outcome for the Nictaba domain which is representative for both proteins (for comparison with the full-length protein see supplementary Fig. A2.1A and Fig. A2.3). Carbohydrate structures with highest reactivity are marked in Fig. 2.4B with their glycan ID and a color corresponding to the bars as shown in Fig. 2.5, which presents interaction for the top 10 glycan structures for each glycan dilution with highest reactivity on the array. These results demonstrate that F-box-Nictaba and its Nictaba-like domain preferentially bind Gal-containing motifs including 6^2-β-D-galactosyl-β1-4-D-galactotriose, 6^2-α-D-galactosyl-β1-4-D-galactotriose and 4^2,6^2-α-D-digalactosyl-β1-4-D-galactobiose with strongest affinity to 6^2-β-D-galactosyl-β1-4-D-galactotriose. This carbohydrate structure presents the highest signal intensities in almost all concentrations and is the only glycan still detectable in two technical replicates in the spots with the lowest glycan concentration (Fig. 2.5C and Fig. A2.3B). Reactivity of the recombinant proteins with D-galactose, β1-4-D-galactobiose and β-1-4-D-galactopentose (glycan IDs 65-67) is only detectable for the spots with the highest glycan concentration and their signal intensities are below the score of top 40 carbohydrates (results not shown). This suggests that F-box-Nictaba shows the strongest affinity towards a specific arrangement of β1-4-linked Gal units. Apart from the clear preference for Gal-containing structures, a lower signal is also detected for feruloylated α1-5-L-arabinobiose/triose and glucose.

Probing the plant glycan array with the Nictaba protein from tobacco confirmed previous studies reporting its specificity towards GlcNAc oligomers (Chen *et al.,* 2002; Gheysen, 2011; Lannoo *et al.,* 2006b). As depicted in supplementary Fig. A2.1B and Fig. A2.4 Nictaba shows the highest affinity for hexaacetyl-chitohexaose ($GlcNAc_6$) and the signal intensity for interaction gradually drops with decreasing number of GlcNAc units as follows: $GlcNAc_6 > GlcNAc_5 > GlcNAc_4$.

It has to be noted that particularly F-box-Nictaba and its Nictaba-like domain, but also to a lesser extent Nictaba from tobacco, were binding to some polysaccharides on the plant glycan array in an

aspecific way. These structures are marked with blue boxes (Fig. 2.4B and Fig. A2.1). These polysaccharides include the gum and β-glucan samples (IDs 8-11 and 22–28) and are reported by the provider to be problematic due to high viscosity, which may create satellites (smaller and unprecise dots). Beacause of this issue, they have been printed on the array at different concentrations than the remaining polysaccharides. Indeed, these so-called satellites become evident following detection. Spots corresponding to these glycans are also clearly visible already on the non-probed arrays presented in supplementary Fig. A2.2A (but not detectable in the negative control, Fig. A2.2B), suggesting that they might interact aspecifically with the lectins. For this reason these polysaccharides have been excluded from the analysis.

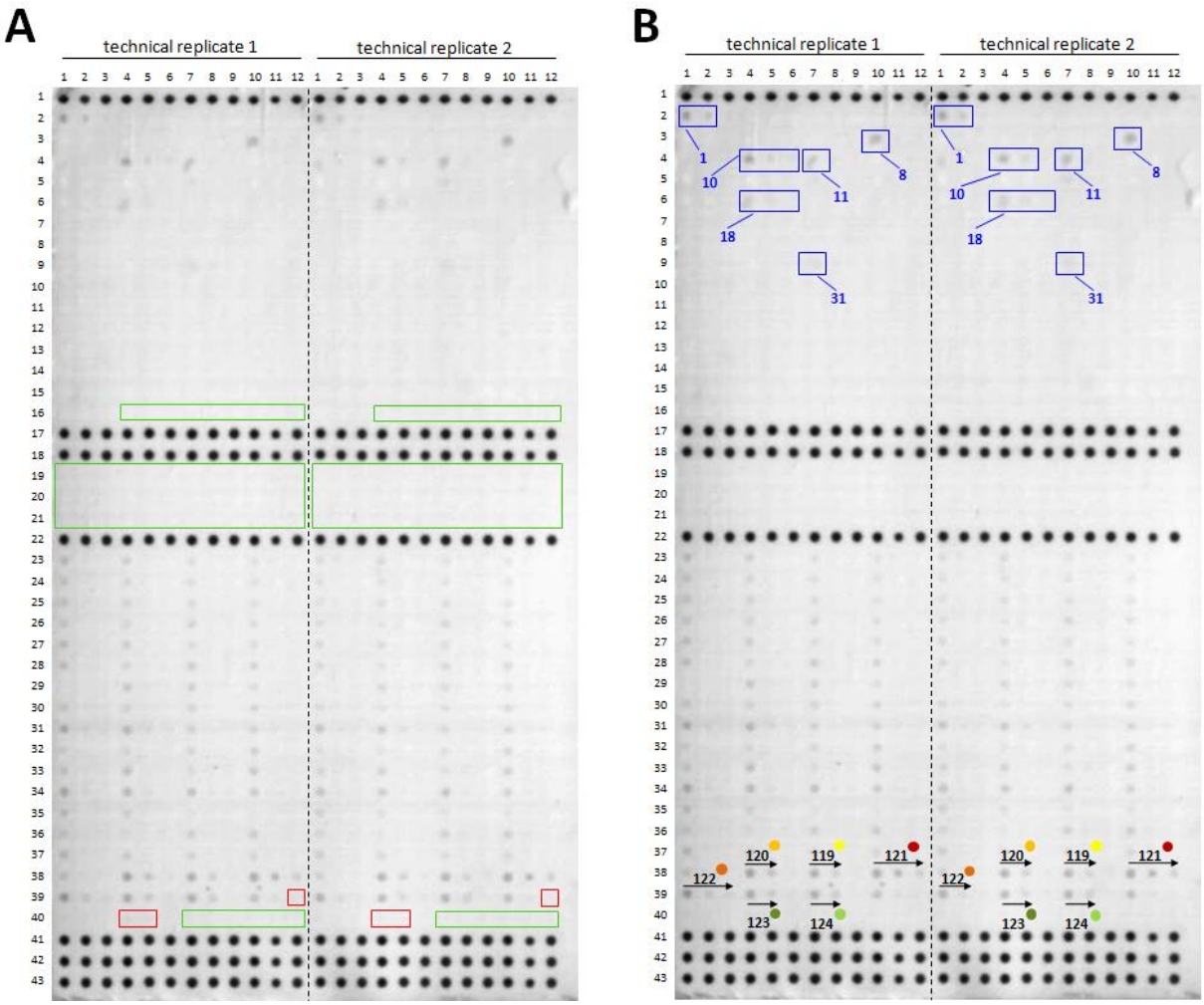


Fig. 2.4 Grayscale images of the scanned plant glycan microarray probed with a recombinant Nictaba-like domain of F-box-Nictaba from Arabidopsis (10 µg/ml) and immunodetected as described in Materials and methods. A, Red boxes mark negative controls (BSA); green boxes mark blanks. B, Glycan structures of highest reactivity are marked with their glycan ID and colors corresponding to the bars in Fig. 2.5. Arrows indicate decreasing concentration of the detected glycan sample. Blue boxes mark those glycans for which the reaction appears to be aspecific.

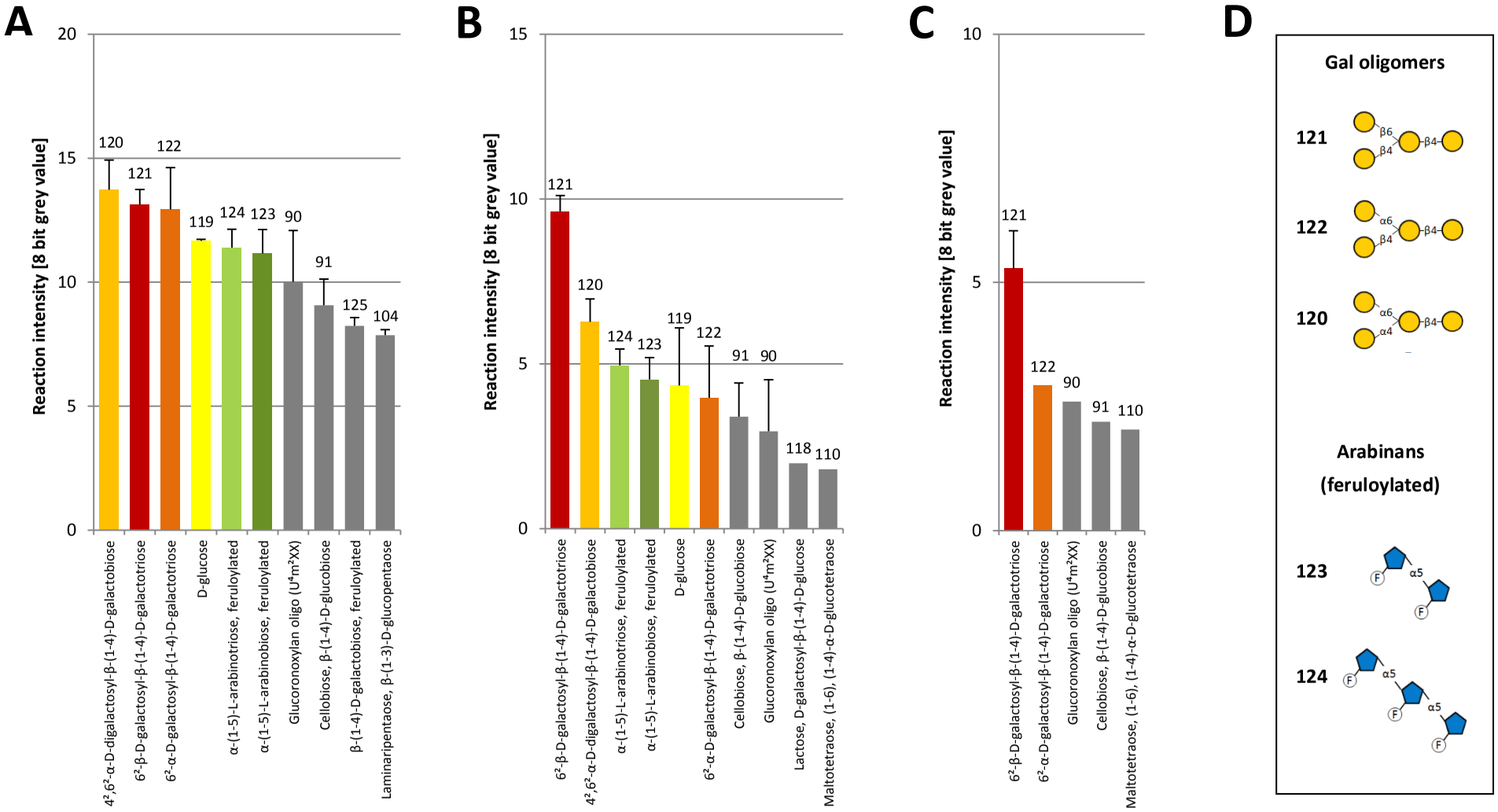


Fig. 2.5 Result of the plant glycan array screening with the Nictaba domain of F-box-Nictaba from *A. thaliana* tested at 10 µg/ml. Results are shown for glycans printed at (A) highest (2 mg/ml), (B) intermediate (0.4 mg/ml) and (C) lowest (0.08 mg/ml) concentration. Reaction intensities are presented for the top 10 glycan structures for each glycan dilution with highest reactivity on the array. For glycans detectable in two technical replicates, bars represent their mean value with SD. Colors are representing glycans as in Fig. 2.4B. D, Graphical representation of glycans with highest reactivity on the array.

2.5 Discussion

2.5.1 F-box-Nictaba is a functional lectin

Judging from the results of the ovomucoid affinity chromatography, the agglutination assays and the glycan array analysis it can be concluded that the Arabidopsis F-box-Nictaba protein encoded by *At2g02360* is a functional lectin. Parallel experiments with the Nictaba domain demonstrated that the lectin activity of At2g02360 resides in its C-terminal Nictaba domain. Based on the high sequence similarity between the C-terminal Nictaba domain of the Arabidopsis protein At2g02360 and Nictaba from tobacco leaves it was tempting to speculate that the F-box-Nictaba protein exhibited the same or at least similar glycan-binding properties as Nictaba and accordingly would interact preferentially with GlcNAc oligomers, and high-Man and complex *N*-glycans. However, glycan array analysis clearly demonstrated that the Arabidopsis F-box-Nictaba protein exhibits a substantially different carbohydrate-binding specificity. Based on the CFG glycan microarray analysis F-box-Nictaba recognizes type 1 and type 2 LacNAc, type 2 poly-LacNAc, Lewis A, Lewis X, Lewis Y, and type-1 B antigen motifs. Screening of the plant glycan microarrays revealed that F-box-Nictaba can interact with Gal oligomers composed of more than two specifically arranged β1-4-linked Gal units. F-box-Nictaba also binds to feruloylated α1-5-L-arabinobiose/triose glycans, though with lower affinity. It should be noted that the Gal-containing motifs including 6^2-β-D-galactosyl-β1-4-D-galactotriose, 6^2-α-D-galactosyl-β(1-4-D-galactotriose and 4^2,6^2-α-D-digalactosyl-β1-4-D-galactobiose which gave the strongest interaction signal on the plant-specific arrays, represent carbohydrate structures which were chemically synthesized and are not known to occur in plants (Pedersen *et al.*, 2012). However, the feruloylated α1-5-linked arabino-oligosaccharides are of plant origin as they have been prepared by enzymatic digestion of sugar beet pulp (Ralet *et al.,* 1994). Altogether, these glycan array data indicate that F-box-Nictaba preferentially binds glycan motifs comprising (a) terminal Gal unit(s) linked to GlcNAc or other Gal residues.

It is intriguing that both recombinant proteins (full-length F-box-Nictaba and its Nictaba domain) are capable of agglutinating red blood cells. This property requires a multivalent lectin, whereas F-box-Nictaba comprises a single carbohydrate-binding domain. This observation, altogether with the fact that recombinant F-box-Nictaba secreted by *P. pastoris* occurs as a protein of much higher MW than expected, points towards the possibility of F-box-Nictaba dimerization. Within the AA sequence of F-box-Nictaba there are eight Cys residues which could contribute to disulphide bond formation. This seems particularly possible in a concentrated protein solution (e.g. crude protein extract of *P. pastoris* medium or concentrated pure protein fraction), where self-association of proteins is strongly enhanced (Snoussi and Halle, 2005). Strikingly, several F-box proteins in yeast and mammals have been demonstrated to form dimers (Hao *et al.,* 2007; Kominami *et al.,* 1998; Li and Hao, 2010; Suzuki *et al.,* 2000; Tang *et al.,* 2007; Welcker and Clurman, 2007). Certainly this issue should be studied

further, particularly with regard to the potential consequences of F-box-Nictaba dimerization for its functionality.

2.5.2 Occurrence of LacNAc motifs

In the last few years LacNAc structures have been studied most intensively in higher animals where they are responsible for blood group determination, cell-to-cell recognition and adhesion processes (Stanley and Cummings, 2009). Different glycans containing LacNAc motifs have been also found in bacteria and viruses (Monzavi-Karbassi *et al.,* 2004; Preston *et al.,* 1996; Wang *et al.,* 2000). However, in plants only Lewis A motifs have been identified so far. These structures are localized at the cell surface (membrane-bound) or can be found in glycoproteins secreted by plant cells or in the Golgi apparatus where they are synthesized (Fitchette *et al.,* 1999; Fitchette-Lainé *et al.,* 1997; Maeda and Kimura, 2014; Maeda *et al.,* 2010; Melo *et al.,* 1997). Although Lewis A structures are widespread within the plant kingdom including monocots, dicots and gymnosperms there has been some controversy to the presence of Lewis A motifs in *A. thaliana* and other members of the Brassicaceae family (Fitchette *et al.,* 1999; Fitchette-Lainé *et al.,* 1997; Rayon *et al.,* 1999; Wilson *et al.,* 2001). Nevertheless, it has been unambiguously shown that *A. thaliana* contains the indispensable enzymatic machinery and can synthesize Lewis A structures. Léonard *et al.* (2002) demonstrated that *A. thaliana* possesses an active α1,4-fucosyltransferase (FUT13) capable of α1,4-fucosylation of the GlcNAc residue within the Galβ1-3GlcNAc structures. These Lewis A epitopes were detected in the plasma membrane and the Golgi vesicles of *A. thaliana* cells. Furthermore, Strasser *et al.* (2007) reported the presence and activity of the β1,3-galactosyltransferase (GALT1) in *A. thaliana* responsible for the formation of Galβ1-3GlcNAc, a structure required for the synthesis of Lewis A structures. It has also been shown that the expression of Lewis A motifs in *A. thaliana* is tissue specific with relatively high levels in pedicels, stems and nodes, moderate levels in siliques and shoot apex, and relatively low levels in flowers and roots, whereas it was not detectable in leaves, which could explain previous problems in the detection of this glycan motif. Therefore, it seems evident that *A. thaliana* synthesizes Lewis A structures, however, most probably at substantially lower levels than other plants and/or the expression of the glycosyltransferases involved (i.e. β1,3-galactosyltransferase and α1,4-fucosyltransferase) is tissue and/or time specific. Unlike the type 1 LacNAc (Galβ1-3GlcNAc) and Lewis A motifs, the other structures recognized by the Arabidopsis F-box-Nictaba protein on the glycan array have not been reported in plants. However, this is not surprising since plants are missing the gene encoding β1,4-galactosyltransferase, the enzyme crucial for the biosynthesis of Galβ1-4GlcNAc structures (Bakker *et al.,* 2001). As a consequence, neither type 2 poly-LacNAc nor Lewis X epitopes can be synthesized by plants. Several research groups have shown that transgenic lines in which the human or rat β1,4-galactosyltransferase gene was introduced into the plant genome are capable of producing the mammalian-type Lewis structures (Karg *et al.,* 2010; Rouwendal *et al.,* 2009). Similarly, up till now no structures related to blood group B antigens could be identified in plants, which most probably also results from the lack of the

necessary enzyme, the α1,3-galactosyltransferase. Clearly, no putative α1,3-galactosyltransferase gene has been identified in the genome of *A. thaliana.*

2.5.3 Occurrence of feruloylated α1-5-arabino-oligosaccharides

Arabino-oligosaccharides are components of plant cell wall polymers including hemicelluloses and pectins (Caffall and Mohnen, 2009; Harholt *et al.,* 2010; Scheller and Ulvskov, 2010). Feruloylated α1-5-arabinans are mostly reported as side chains α1-4-linked to rhamnose residues in the rhamnogalacturonan I (RGI) backbone of pectins, forming the branched RGI structure in plant cell walls. The substitutions with ferulic acid can either be present as monomers or as dimers through oxidative coupling with other side chains and as such can cross-link pectin molecules (Levigne *et al.,* 2004; Ralet *et al.,* 2005; Waldron *et al.,* 1997). Structurally heterogeneous feruloylated arabino-oligosaccharide side chains of RGI have been well described in different plant species, especially in sugar beet (Levigne *et al.,* 2004; Ralet *et al.,* 1994, 2005; Sato *et al.,* 2013), spinach (Ishii and Tobita, 1993), potato (Bush *et al.,* 2001; ØBro *et al.,* 2004), soybean (Huisman *et al.,* 2001; Nakamura *et al.,* 2002) and apple (Peña and Carpita, 2004). The α1-5-linked arabino-oligosaccharides have also been identified in the Arabidopsis cell walls (Pettolino *et al.,* 2012; Verhertbruggen *et al.,* 2009, 2013). The synthesis of feruloylated α1-5-linked arabinose oligomers requires the activity of α1,5-Ara-transferases (AraT) and feruloyl transferase (Caffall and Mohnen, 2009). One putative α1,5-AraT has been identified in Arabidopsis, called ARABINAN DEFICIENT1 (ARAD1) encoded by *At2g35100* (Harholt *et al.,* 2006), which is a type II membrane protein localized in the Golgi apparatus. It has been demonstrated that loss-of-function *arad1* mutants present a reduced arabinan content in the cell wall. Next to ARAD1, 7 additional Arabidopsis homologs have been found (Harholt *et al.,* 2006). Genes involved in feruloylation of cell wall components have been identified in rice (Mitchell et al., 2007; Piston et al., 2010), but no feruloyl transferase responsible for arabinan feruloylation has been identified in *A. thaliana*.

2.5.4 Other lectins recognizing β-galactosides

A well-known group of lectins with specificity towards β-galactosides such as [Gal(NAc)β1-3/4GlcNAc] are the galectins. Galectins are widely expressed and have been found in mammals, birds, insects, fish, nematodes, sponges and some fungi. Galectin-like sequences have also been predicted in the genome of the plant *A. thaliana* (Cooper and Barondes, 1999; Cummings and Liu, 2009). Most members of the galectin family are able to interact with simple β-galactosides such as di- or tri-saccharides, but their affinity is relatively weak. In contrast, galectin binding to natural β-galactoside-containing glycoconjugates is of much higher affinity (Cummings and Liu, 2009). To date, the human galectin family comprises 15 members, each with different sugar specificity towards the di-saccharide LacNAc, poly-LacNAc and internal LacNAc present in poly-LacNAc (Stowell *et al.*, 2008).

Several members of the galectin family are primarily located in the nucleus and the cytoplasm of cells. Within the cytoplasm there appears to be a selective targeting of the individual galectins to sub-compartments of the cytoplasm, to sub-cellular organelles and to sub-regions within membranes (Liu *et al.*, 2002). Although galectins lack a classical signal sequence and are synthesized on free ribosomes, they can also be secreted by non-classical export to the outer plasma membrane and extracellular matrix (Delacour *et al.*, 2009; Schneider *et al.*, 2010). As a consequence, the same galectin can have a dual localization inside as well as outside cells depending on the cell type where it is expressed (Arnoys and Wang, 2007; Newlaczyl and Yu, 2011). A common feature of secreted (soluble) galectins is the cross-linking of glycoconjugate ligands located at the cell surface and within the extracellular matrix. This cross-linking is based on the interaction of the galectins with LacNAc structures decorating the extracellular ligands. Through this mechanism, galectins modulate important processes such as cell adhesion, migration, polarity, chemotaxis, inflammation, proliferation and apoptosis (Bi *et al.*, 2011; Boscher *et al.*, 2011; Garner and Baum, 2008; Liu and Rabinovich, 2010; Vasta, 2009). Secreted galectins as well as intracellular galectins also deliver a variety of intracellular signals to the relevant intracellular signal-regulation pathways to modulate mitosis, apoptosis and cell-cycle progression. Important to note is that unlike the secreted galectins, the galectins present in the nucleus and the cytoplasm commonly interact with their intracellular ligands based on protein-protein interactions rather than using lectin-glycan interactions (Liu *et al.*, 2002). However, as was reported for galectin-3, galectins need a functional carbohydrate-binding domain for proper protein interactions with a redundant pre-mRNA splicing factor shuttling between the nucleus and cytoplasm, and the addition of LacNAc or lactose can perturb some of these interactions (Yang *et al.*, 1996). According to Haudek *et al.* (2010) studying the dynamics of galectin-3, exportins and importins are used to target the galectin to its proper sub-compartment. However, mutational analysis of the carbohydrate-binding domain of galectin-3 or addition of LacNAc can perturb this protein-protein interaction based targeting and may even affect apoptosis of cells (Gaudin *et al.*, 2000; Salomonsson *et al.*, 2010). Hence, the significance of the LacNAc-binding activity of galectins remains a challenge to be explored in more detail.

Outside the galectin family, also other lectins have been described to interact with LacNAc containing sugars and glycoproteins. One fungal (cytoplasmic) ricin-B like lectin from the mushroom *Clitocybe nebularis* was recently shown to recognize LacdiNAc (Pohleven *et al.*, 2012). Furthermore, several plant lectins have been reported to interact with LacNAc structures, including the lectin from *Arum maculatum* (Allen, 1995), the *Erythrina cristagalli* lectin (Teneberg *et al.*, 1994), and PHA-E/L from *Phaseolus vulgaris* (Kaneda *et al.*, 2002). All these lectins are located in the plant vacuole and hence can be used as defense proteins. Recent evidence also supports the presence of a LacNAc binding lectin in the nucleocytoplasmic compartment of the plant cell, such as the EULS3 protein from *A. thaliana* (Van Hove *et al.*, 2011) and the jacalin-related lectins from rice (Orysata), *Calystegia sepium* (Calsepa) and *Morus nigra* (Morniga M) (Al Atalah *et al.*, 2011).

2.5.5 Relevance of plant lectin F-box-Nictaba

This is the first report of the characterization of a sugar-binding F-box protein in plants. Sugar-binding F-box proteins are already known since 2002, when they were first discovered in mammals (Yoshida and Tanaka, 2010). Unlike the plant Fbs family, the mammalian Fbs protein family is a very small group consisting of only 5 homologous proteins, referred to as FBG1-FBG5. FBG1 and FBG2 exhibit carbohydrate-binding specificity towards high-Man *N*-glycans similar to the glycan specificity reported for Nictaba (Glenn *et al.,* 2008; Yoshida *et al.,* 2005). Both FBG1 and FBG2 have been proposed to play a role in protein quality control by recognizing and targeting misfolded or incompletely assembled glycoproteins for degradation through the ERAD pathway (Yoshida and Tanaka, 2010). Based on the striking similarities between the Fbs proteins and Nictaba for what concerns their localization pattern in the nucleus and cytoplasm of the cell, the three-dimensional conformation of the lectin domain and the carbohydrate-binding properties the hypothesis was put forward that the nucleocytoplasmic Arabidopsis F-box-Nictaba protein might be a functional homolog of the mammalian FBG1 and FBG2 proteins in plants (Lannoo *et al.,* 2008). However, the glycan arrays show that the plant F-box-Nictaba protein encoded by *At2g02360* recognizes and binds Gal oligomers as well as type 1 LacNAc, Lewis A structures, type 2 poly-LacNAc, Lewis X and Y epitopes and blood group B antigens. As such, the sugar-binding specificity of the plant Fbs protein resembles better the one reported for the mammalian Fbs proteins FBG4 and FBG5, which exhibit strong affinity towards sulfated glycan structures and different glycans with type 2 LAcNAc (Galβ1-4GlcNAc) motifs (see supplementary Table A2.4). Supplementary Table A2.4 gives an overview of the glycan-binding properties of the members of the mammalian Fbs protein family. Comparative analysis shows that within the mammalian Fbs protein family there is a wide divergence in carbohydrate-binding activity. While the mammalian Fbs protein FBG1 is highly specific to high-Man *N*-glycans, FBG2 strongly binds not only high-Man but also complex *N*-glycans and sulfated glycan structures. FBG3 does not exhibit carbohydrate-binding activity at all. Hence, although the mammalian Fbs proteins share high sequence similarity, they differ substantially in their glycan-binding properties. Due to the diversity in carbohydrate-binding specificity the different Fbs proteins are suggested to play divergent roles in the glycome regulation in mammals (Glenn *et al,* 2008). Likewise, it seems that the carbohydrate-binding site of F-box-Nictaba has developed a different specificity from the one of the Nictaba protein from tobacco, despite the similarity at the sequence level. Taken into account that the Arabidopsis genome contains a whole family of homologous F-box-Nictaba proteins which slightly differ in the sequences of the Nictaba domains it is likely that these chimera proteins show broad differences in their fine specificities. Therefore it cannot be excluded that other plant F-box-Nictaba proteins might have carbohydrate-binding properties that are more similar to that of Nictaba.

What is more, the demonstration of differences in specificity between Nictaba and F-box-Nictaba, indicating gene divergence within the family of Nictaba-related lectins, urges for extreme caution when making predictions regarding the specificity of lectins. Previously it was also shown that gene divergence within the legume lectin family (Loris *et al.,* 1998), the jacalin-related lectins (Rougé et al.,

2003), the GNA-related lectins (Fouquaert *et al.,* 2009b) and recently also within the EUL family (Van Hove *et al.,* 2011) has resulted in changes in carbohydrate-binding specificity. Thus, although molecular modeling of the lectin structure and the glycan-binding site is possible, it is a highly complex procedure and certainly does not allow to draw conclusions regarding the specificity of lectins solely based on their AA sequences. For instance, molecular modeling of the EUL domains from different types of EUL-related proteins sharing high sequence similarity with the lectin domain, showed a very similar fold with conserved AAs in the binding site. Despite that, however, glycan microarray screening revealed that the glycan-binding site of the EUL domain from different EUL-like proteins can accommodate distinct carbohydrate structures, including high-Man *N*-glycans, the type-1 B antigen and Gal-containing motifs (Fouquaert and Van Damme, 2012).

Chapter 3

F-box-Nictaba from *Arabidopsis thaliana* is a plant defense protein involved in pathogen infection and heat stress

Part of the manuscript in preparation:

Stefanowicz K, Lannoo N, Zhao Y, Eggermont L, Van Hove J, Al Atalah B, Rydahl MG, Willats WGT, Van Damme EJM. F-box-Nictaba from *Arabidopsis thaliana* is a plant defense protein involved in pathogen infection and heat stress.

3.1 Abstract

Within the genome of *Arabidopsis thaliana*, a small group of F-box proteins has been identified consisting of an F-box domain linked to a domain homologous to the glycan-binding protein Nictaba. Nictaba is a GlcNAc-binding protein originally identified in tobacco leaves, which expression is up-regulated after jasmonate treatment and insect herbivory and which has been shown to increase the plant's resistance towards insects. In Chapter 2, the Arabidopsis F-box-Nictaba protein, encoded by the gene *At2g02360*, has been shown to be a functional lectin that can bind *N*- and *O*-glycans containing *N*-acetyllactosamine (LacNAc) structures, Lewis A, Lewis X, Lewis Y and type-1 B antigen motifs.

Here, we present a detailed qRT-PCR expression analysis of *At2g02360* in *A. thaliana* plants upon different stresses and hormone treatments. The expression of the *F-box-Nictaba* gene was enhanced after plant treatment with the defense-related plant hormone salicylic acid (SA) and after plant infection with the virulent *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000). Histochemical staining of Arabidopsis plants expressing a *pAt2g02360:GUS* reporter construct displayed preferential activity of the *At2g02360* promoter sequence in non-glandular trichomes present on young rosette leaves. Meta-analysis database searches revealed co-expression of *At2g02360* with genes involved in disease and plant defense responses. Transgenic *A. thaliana* plants impaired in *F-box-Nictaba* gene expression and plants overexpressing *F-box-Nictaba* showed differential gene expression in comparison to wild type (WT) plants during heat stress and after *Pst* DC3000 infection. Moreover, *A. thaliana* plants overexpressing the F-box-Nictaba protein demonstrated reduced disease symptoms after *Pst* DC3000 infection. Taken together, our data suggest that this Arabidopsis *F-box-Nictaba* gene is involved in SA-related plant defense responses.

3.2 Introduction

F-box proteins represent one of the largest and most diverse protein families with more than 700 members in the plant kingdom (Gagne *et al.*, 2002; Jain *et al.*, 2007; Hua *et al.*, 2011). They are named after their highly conserved N-terminal protein-protein interaction motif of approximately 50 AA residues, known as the F-box domain. The majority of the F-box proteins function as part of SCF-type Ub E3 ligases (Petroski and Deshaies 2005) in which the F-box protein comprises the substrate-binding module. F-box proteins are assembled into active SCF complexes through a direct binding of the F-box motif with the SCF core protein Skp1 (S-phase kinase-related protein 1). Through their variable C-terminal substrate-binding domain, F-box proteins bind specifically to and deliver appropriate substrates to the SCF complex for ubiquitin (Ub)-mediated proteolysis by the ubiquitin-proteasome system (UPS) (Skaar *et al.*, 2013) (Chapter 1, Section 1.2).

The impressive number of F-box proteins in plants (especially when compared to fungi and animals) and the extensive diversity of their C-terminal target-binding domains contribute to the ability of SCF

complexes to target a wide variety of substrates. Therefore, it is not surprising that F-box proteins are involved in numerous cellular processes within plant development and stress signaling. Genetic approaches already revealed an essential role for F-box proteins in plant hormone perception and signaling in case of ABA, auxin, ET, GA as well as MeJA (Kelley and Estelle 2012). In addition, F-box proteins were reported to be involved in circadian clock control, photomorphogenesis and flowering (Somers *et al.*, 2004; Lechner *et al.*, 2006; Ito *et al.*, 2012), leaf senescence (Woo et al. 2001), self-incompatibility (Qiao *et al.*, 2004a,b) and responses to various (a)biotic stresses (Calderon-Villalobos *et al.*, 2007; Zhang *et al.*, 2008; Cheng *et al.,* 2011; Bu *et al.*, 2013).
F-box proteins recognize their substrates in several ways. In most cases, the F-box proteins contain C-terminal protein-protein interaction motifs such as LRR and WD-40 repeats that bind phosphorylated proteins. These F-box proteins are referred to as FBXL and FBXW proteins, respectively. However, phosphorylation-based substrate-binding is not the only mechanism for F-box proteins to recruit a target for degradation. Recent analyses have shown that multiple newly discovered F-box proteins (the so-called FBXO proteins) combine various recognition mechanisms which enable tight regulation of substrate selection by the F-box protein and each complementary SCF complex (Skaar *et al.*, 2013). Amongst these new FBXO proteins, glycan-binding F-box proteins were first discovered in mammals about 10 years ago (Yoshida *et al.*, 2002; Yoshida and Tanaka 2010). Recently, they were also reported in plants with the finding of F-box-Nictaba proteins, consisting of an F-box domain linked to a glycan-binding domain resembling the tobacco lectin Nictaba (Lannoo *et al.*, 2008; Stefanowicz *et al.*, 2012) (see Chapter 2).

In the mouse and human genome, at least five *Fbs* genes have been identified (Yoshida and Tanaka 2010), among which FBG1, FBG2 and FBG5 which bind to high-Man *N*-glycosylated proteins (Glenn *et al.,* 2008) (supplementary Table A2.4). NMR studies showed that the C-terminal substrate-binding domain of both FBG1 and FBG2 specifically interacts with the inner N,N'-diacetylchitobiose ($GlcNAc_2$) core of high-Man *N*-glycans present on incompletely folded or denatured glycoproteins. Since FBG1 and FBG2 do not target free Man structures or non-glycosylated proteins, it was concluded that both Fbs proteins function as glycan-binding F-box proteins in SCF complexes involved in the ERAD pathway (Yoshida *et al.*, 2005). In this pathway, proteins which fail to fold correctly or assemble into oligomeric complexes in the lumen of the ER are retro-translocated to the cytosol, where they are captured by an $SCF^{FBG1/2}$ complex before degradation by the UPS (Hoseki *et al.*, 2010).

The genome of *A. thaliana* contains multiple genes encoding F-box proteins with a putative glycan-binding or lectin-like domain. These proteins are referred to as the Arabidopsis F-box-Nictaba family, since their C-terminal domain highly resembles the *N*-glycan-binding jasmonate-inducible tobacco lectin Nictaba (Lannoo *et al.*, 2008; Delporte *et al.*, 2015). This plant F-box protein family groups approximately 30 members which all share over 90% and 40-64% sequence similarity in the F-box domain and the Nictaba domain, respectively. Due to the presence of the F-box domain linked to a lectin-like C-terminal substrate-binding domain, it is tempting to speculate that these F-box-Nictaba proteins also function as substrate adaptors in an ERAD-like degradation pathway in plants similar to

the mammalian Fbs proteins. The three-dimensional model of the tobacco protein Nictaba (Schouppe *et al.*, 2010) shows striking similarity to the β-barreled structure of the C-terminal lectin domain of the mammalian FBG1 protein (Mizushima *et al.*, 2004). Furthermore, both Nictaba and FBG1 show comparable glycan-binding properties towards the inner core structure of *N*-glycans (Lannoo *et al.* 2006; Glenn *et al.*, 2008). However, detailed analysis of the sugar specificities for the Arabidopsis F-box-Nictaba protein with the highest sequence homology to Nictaba revealed the unexpected binding of its Nictaba domain to *N*- and *O*-glycans containing (poly)*N*-acetyllactosamine (LacNAc) (Galβ1-3GlcNAc and Galβ1-4GlcNAc) structures, Lewis A (Galβ1-3(Fucα1-4)GlcNAc), Lewis X (Galβ1-4(Fucα1-3)GlcNAc) and Lewis Y (Fucα1-2Galβ1-4(Fucα1-3)GlcNAc) motifs, as well as to β1-4-linked Gal oligomers and feruloylated α1-5-L-arabinobiose/triose glycans. Only minor interaction was reported with $Man_3GlcNAc_2$ and $Man_{5-8}GlcNAc_2$ *N*-glycans (Stefanowicz *et al.,* 2012; Chapter 2).

To determine whether the Arabidopsis F-box protein encoded by *At2g02360* is involved in stress signaling pathways, wild type Arabidopsis plants were treated with a wide range of (a)biotic stress factors (including plant hormones, heat, cold, osmotic stress and pathogen infections) and an extensive expression profiling analysis was performed to identify changes in gene expression after different treatments. Quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR) revealed an up-regulation of F-box-Nictaba gene expression after SA treatment and *Pseudomonas syringae* pv. *tomato* strain DC3000 infection. β-glucuronidase (GUS) histochemical assays showed preferential activity of the *At2g02360* promoter sequence in leaf trichomes of transgenic Arabidopsis plants. When compared to WT plants, *A. thaliana* plants overexpressing the F-box-Nictaba protein exhibited a lower degree of leaf damage after infection with *Pst* DC3000 and a higher expression of the *WRKY70* gene encoding a SA-related transcription factor. Transgenic Arabidopsis plants with either reduced or enhanced F-box-Nictaba expression also exhibited differential expression of the *Hsp70b* gene during heat stress, when compared to WT plants. The *F-box-Nictaba* gene expression levels themselves were also affected in the transgenic plants by different stress conditions. Altogether, our data suggest a role for the F-box lectin protein in plant defense-related pathways.

3.3 Materials and methods

3.3.1 Plant materials and growth conditions

Seeds of WT *A. thaliana* ecotype Columbia-0 (Col-0) were purchased from Lehle Seeds (Round Rock, Texas, USA). Seeds of SALK T-DNA insertion mutant lines associated with the *At2g02360* locus SALK_007866 and SALK_085735C (further referred to as knockout lines KO4 and KO6, respectively) (Alonso *et al.,* 2003), were obtained from the European Arabidopsis Stock Centre (NASC, University of Nottingham, UK).

To establish *in vitro* cultures, dry seeds were surface sterilized by washing the seeds for 4 min in 70% (v/v) ethanol and 10 min in 6% (v/v) bleach, followed by rinsing three to four times with sterile distilled water. Sterilized Arabidopsis seeds were sown on sterile filter paper which was placed on top of solid Murashige and Skoog (MS) medium (Duchefa, Haarlem, The Netherlands). To establish non-*in vitro* grown plants, Arabidopsis seeds were directly sown into artificial soil (Jiffy-7, 44 mm Ø) (AS Jiffy Products, Drobak, Norway) or into expanded clay granules (> 4mm Ø). To break dormancy, the seeds were stratified at 4°C for 3 days in the dark. Afterwards, seeds were transferred to a controlled growth chamber set at 21°C with a 16/8 h light/dark photoperiod for seed germination and plant development. Only for infection experiments, seeds were kept in a growth chamber (Conviron Germany GmbH, Berlin, Germany) set at 21°C with a 12/12 h light/dark photoperiod. All trays containing seedlings and plants grown in artificial soil or granules were watered regularly to keep them moist. For *At2g02360* gene expression analysis in WT *A. thaliana* plants (Col-0) grown under standard growth conditions, plant samples were collected from different developmental stages as defined by Boyes *et al.* (2001). Early stage plant materials (cotyledons, 4 leaves and 8 leaves stage) were collected from *in vitro* grown plants and included the complete seedlings, whereas the other samples were taken from plants grown in artificial soil. Root material was collected from plants grown in granules.

3.3.2 Chemical reagents

6-benzylaminopurine (BAP), gibberellic acid (GA_3) and MeJA were purchased from Sigma-Aldrich (Bornem, Belgium). ABA and ethephon were obtained from Acros Organics (Geel, Belgium). SA, IAA and salt (NaCl) were obtained from Duchefa, whereas mannitol was purchased from VWR (Leuven, Belgium). MG132 was obtained from Enzo Life Sciences (Antwerpen, Belgium). Prior to use, appropriate amounts of MeJA, SA, ABA, IAA and GA_3 were dissolved in 100% ethanol, whereas BAP and MG132 were dissolved in 100% dimethyl sulfoxide (DMSO) (VWR). Ethephon, NaCl and mannitol were dissolved in water.

3.3.3 Hormone treatments and abiotic stress application

In vitro grown 16-day-old seedlings were used. The filter papers containing germinated seedlings were carefully transferred from the MS agar plates to Petri dishes filled with liquid MS medium containing either a hormone solution (100 µM concentration in case of ABA, BAP, ethephon, GA_3, IAA or MeJA and 300 µM concentration in case of SA), 50 µM MG132, 150 mM NaCl or 100 mM mannitol and incubated at 21°C for appropriate times. Controls were kept on liquid MS medium containing an equal volume of the corresponding solvent (ethanol, DMSO or water). Cold and heat stress were applied by incubating the MS agar plates with the seedlings in the dark either at 4°C or 37°C, respectively. Concomitant controls were incubated at 21°C in the dark. For every stress application

20-30 seedlings were collected for RNA extraction at several time points after stress initiation, frozen in liquid nitrogen immediately and stored at -80°C prior to RNA extraction and qRT-PCR analysis.

3.3.4 Infection assays

Both the *Pseudomonas syringae* pv. *tomato* strain DC3000 and the *Botrytis cinerea* strain B05.10 were a kind gift from Prof. dr. M. Höfte (Phytopathology, Ghent University, Belgium). Infection assays were performed as described elsewhere (Pieterse *et al.*, 1996, Katagiri *et al.*, 2002; Audenaert *et al.*, 2002) with some minor modifications.

The *Pseudomonas* bacteria were grown in liquid King's B medium at 28°C at 200 rpm till the culture reached the mid to late log phase growth (OD_{600} = 0.6 – 1.0). Then, the culture was centrifuged at 2500 g for 10 min. Bacterial cells were re-suspended in 10 mM $MgSO_4$ to obtain a bacterial solution of OD_{600} = 0.05, corresponding to 2.5 x 10^7 cfu /ml of *Pseudomonas* bacterial cells. This infection solution was supplemented with 0.05% Silwet-77 (GE Specialty Materials (Suisse) S.a.r.l., Switzerland) prior to use. A mock solution consisted of 10 mM $MgSO_4$ supplemented with 0.05% Silwet-77. The *Botrytis* strain was maintained on regular potato dextrose agar plates at 21°C. To stimulate its sporulation, the plates were incubated for 10 days at 21°C under a 12/12 h UV/dark light regime. At the day of infection, *Botrytis* spores were harvested from 10-day-old cultures by washing the plates with distilled water containing 0.01% Tween-20 (VWR). After filtration over a nylon membrane (20 µm Ø), the conidia were counted using a Bürker counting chamber. An inoculation solution containing 5 x 10^5 conidia / ml was prepared in ½ strength potato dextrose broth medium. The mock solution consisted of the same components without spores.

One hundred individually grown 5-week-old WT Arabidopsis plants were inoculated with either the infection or the mock solutions by 1) spraying the rosette leaves until run-off in case of infection with *Pseudomonas* or by 2) the droplet technique in case of infection with *Botrytis*. In the latter case, a 10-µl droplet of either the infection or the mock solution was added on the upper side of three randomly chosen rosette leaves from each plant. One day before treatment up till two days after bacterial infection, the plants were maintained at 100% relative humidity to increase the infection efficiency. In the fungal assay, the plants were kept at 100% relative humidity during the entire experiment. During infection, the plants were kept separately in a controlled Conviron growth chamber at 21°C with a 12/12 h light/dark photoperiod. At indicated time points post infection, rosette leaves of 8-10 randomly chosen plants were frozen in liquid nitrogen and stored at -80°C prior to RNA extraction and qRT-PCR analysis.

3.3.5 RNA extraction, cDNA synthesis and RT-PCR analysis

All collected plant samples were ground into a fine powder with a mortar and pestle and RNA extracted using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. To remove

any residual genomic DNA, samples were treated with 2 units of RNase-free DNaseI (Fermentas, St. Leon-Rot, Germany) for 30 min at 37°C. After addition of 2 µl EDTA (25mM), the DNase enzyme was inactivated by incubation at 65°C for 10 min. The RNA concentration and purity were measured with a Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA). First-strand cDNA was synthesized from 1 µg of DNA-free total RNA with 1 µl of 50 µM oligo$(dT)_{20}$ using the M-MLV transcriptase kit (Invitrogen) and then diluted 2.5x with RNAse-free water. cDNA quality was checked by RT-PCR using primers specific for the SUMO-conjugating enzyme *UBC9* gene (supplementary Table S1). Reactions included 1.5 µl cDNA, 1 µl dNTPs (10 mM), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 2 µl 10x KEY buffer (VWR), 0.2 µl Taq-polymerase (VWR) and water up to the volume of 20 µl. PCR conditions were as follows: 5' 95°C – 40x (45'' 95°C – 30'' 57°C – 30'' 72°C) – 5' 72°C. PCR amplification products were verified by gel electrophoresis on a 2.5% agarose gel in 0.5x TAE buffer.

3.3.6 Quantitative RT-PCR (qRT-PCR) analysis

qRT-PCR analyses were performed using the SensiMix SYBR kit (Bioline Reagents Ltd, London, UK). The reaction mixture contained the following components: 1x SensiMix™ SYBR, 2 ng/µl first-strand cDNA and 500 nM of gene-specific forward and reverse primers (primers listed in supplementary Table A3.1) in a total volume of 20 µl. For hormone and stress treatments specific positive control genes were included (according to Goda *et al.*, 2008; Kim *et al.*, 2010b; Nishizawa-Yokoi *et al.*, 2010; Besseau *et al.,* 2012; Espunya *et al.*, 2012) (supplementary Table A3.1). qRT-PCR was carried out in a Rotor-Gene 3000 (Corbett Life Science) using Rotor Discs (Qiagen, Hilden, Germany). The program was as follows: 10' 95°C – 45x (25'' 95°C – 25'' 60°C – 20'' 72°C) – 5' 72°C ending with a melting curve generation (gradual increase of temperature from 72°C to 95°C rising by 1°C / step). The output data were generated by the Rotor-Gene 6 software and the results were statistically analyzed via the REST-384 software (Corbett Research, Pfaffl *et al.,* 2002). For each treatment two independent biological replicates were analyzed with two or three technical replicates. Data normalization of gene expression was performed using the reference genes *PP2A, TIP41 and UBC9* (Czechowski *et al.,* 2005).

3.3.7 Selection of SALK lines with knockout (KO) expression of *At2g02360*

Two lines with a T-DNA insertion site within the exon sequences of the *At2g02360* locus were retrieved from the SALK database (http://signal.salk.edu/cgi-bin/tdnaexpress). Both lines were selected on MS agar plates supplemented with 75 mg/l kanamycin (Duchefa) and tested for homozygosity by PCR on total genomic DNA. For each SALK line a different set of three primers was used, including the left border primer of the T-DNA insertion (LBb1.3) as well as two line-specific primers (the left (LP) and the right genomic primer (RP)) (supplementary Table A3.2). For each line, two PCR reactions were set up with the primer combinations: LP+RP and LBb1.3 + RP. Identical PCR

reactions were performed on genomic DNA exctracted from *A. thaliana* WT plants. The DNA quality was checked by PCR using *ACT2* primers (supplementary Table A3.2). Reactions included 200 ng gDNA, 2 µl dNTPs (10 mM), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 2.5 µl 10x Extra buffer (VWR), 0.125 µl Taq-polymerase (VWR) and water up to the volume of 25 µl. Cycling parameters used were 2' 94°C – 30x (15" 94°C – 30" 52°C – 1' 72°C)– 5' 72°C. Gene expression of *F-box-Nictaba* in SALK lines was analyzed by RT-PCR using primers evd790 and evd791 for amplification of the full-length *F-box-Nictaba* sequence (supplementary Table A3.2) and cDNA quality was checked using *PP2A* primers (supplementary Table A3.1). Cycling parameters used were 2' 94°C – 35x (15" 94°C – 30" 55°C – 1' 72°C)– 5' 72°C. qRT-PCR using *F-box-Nictaba*-specific primers (evd786 and evd787; supplementary Table A3.1) was performed to quantify gene expression levels. Homozygous and confirmed KO plants were used for both thermotolerance and infection experiments.

3.3.8 Construction of vectors for the GUS reporter system and for overexpression of untagged F-box-Nictaba

The *pAt2g02360:GUS* reporter construct as well as the CaMV 35S:*At2g02360* construct were generated using the Gateway™ cloning technology (Invitrogen). A 1806 nt *At2g02360* promoter fragment (including the 5' UTR from *At2g02360*) was amplified by a two-step PCR starting from total genomic DNA extracted from 3-week old WT *A. thaliana* Col-0 plants. The *At2g02360* sequence was amplified by a two-step PCR starting from the cDNA clone BX820545 (INRA, Centre de Toulouse, Unité de Recherche 1258-CNRGV, Castanet-Tolosan Cedex, France). Reactions included 200 ng gDNA or 10 ng pDNA, 2 µl dNTPs (10 mM), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 2.5 µl 10x KEY buffer (VWR), 0.125 µl Taq-polymerase (VWR) and water up to the volume of 25 µl. In the first step, primers were used to generate an *At2g02360* promoter sequence (primers evd555 and evd556) and an *At2g02360* gene sequence (primers evd1046 and evd1047) including parts of the attB1 and attB2 Gateway adaptor sites at their 5' and 3' sequences, respectively (supplementary Table A3.3). Primer sequences for promoter amplification were made based on *At2g02360* gene information as available on the TAIR server (TAIR10). Cycling parameters used were: 2' 94°C – 30x (15" 94°C – 30" 55°C – 2' 72°C) – 8' 72°C for promoter amplification and 2' 94°C – 30x (15" 94°C – 30" 57°C – 2' 72°C) – 5' 72°C for *At2g02360* gene sequence amplification. In the second step, primers evd2 and evd4 were used to complete the attB sites (supplementary Table A3.3). Cycling parameters used were 2' 94°C – 5x (15" 94°C – 30" 50°C – 2' 72°C) – 20x (15" 94°C – 30" 55°C – 2' 72°C) – 8' 72°C for promoter amplification and 2' 94°C – 5x (15" 94°C – 30" 50°C – 2' 72°C) – 25x (15" 94°C – 30" 57°C – 1' 72°C) – 5' 72°C for *At2g02360* gene sequence amplification. AttB-PCR products were then cloned via the pDONR221 donor vector (Invitrogen) into the pKGWFS7.0 or pK7WG2.0 destination vector for promoter and gene sequence, respectively, (Karimi *et al.* 2002) using the Gateway® BP and LR Clonase™ mixes (Invitrogen). The binary vectors containing the *pAt2g02360:GUS* construct or CaMV 35S:*At2g02360* construct were sequenced and introduced into *A. tumefaciens* strain GV3101 using electroporation.

WT Arabidopsis Col-0 plants were transformed using the floral-dip method (Clough and Bent, 1998) Transgenic progenies were selected on MS agar plates supplemented with 75 mg/l kanamycin. Integration of the T-DNA into the plant genome was checked by PCR on genomic DNA extracted from 3-week-old seedlings using GUS-specific primers GUS-F and GUS-RV or kanamycin-specific primers evd463 and evd261 (supplementary Table A3.3). PCR conditions were as follows: 2' 94°C – 25x (15" 94°C – 30" 52°C – 1' 72°C) – 8' 72°C for the *pAt2g02360:GUS* reporter construct or 2' 94°C – 30x (15" 94°C – 30" 52°C – 1' 72°C) – 5' 72°C for the CaMV 35S:*At2g02360* construct. Plants homozygous for the promoter:GUS construct in the T3 generation were used for the histochemical assays. Plants homozygous for the CaMV 35S:*At2g02360* construct (overexpression (OE) lines) in the T4 generation were tested by qRT-PCR and Western blot analysis and used for the thermotolerance and infection experiments.

3.3.9 Histochemical GUS assays

The GUS assay was performed according to Jefferson (1987) with minor adaptations. Arabidopsis seedlings and plants of different ages were first placed in 90% acetone for 30 min at 4°C. After three washes for 5 min in 0.1 M phosphate buffer (pH 7.0), plants were incubated for 30 min at 37°C in GUS pre-incubation buffer (*i.e.* 0.1 M phosphate buffer supplemented with 0.5 mM K-ferricyanide (VWR) and 0.5 mM K-ferrocyanide (VWR)). Afterwards, seedlings were transferred to the GUS assay buffer (*i.e.* GUS pre-incubation buffer supplemented with 2 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-Gluc (Fermentas), dissolved in DMSO)) and incubated overnight at 37°C in the dark. The reaction was stopped by washing the plants three times in phosphate buffer for 10 min. Microscopic analysis was performed on a Nikon eclipse TE2000-e microscope (Nikon Belux, Brussels, Belgium) and a Leica DFC400 microscope (Leica, Heerbrugg, Germany) using the NIS-Elements and Leica Application Suite software packages, respectively.

3.3.10 Bioinformatic analyses

Promoter sequences were scanned for the presence of *cis*-elements identical or similar to motifs registered in the two plant *cis*-acting regulatory elements databases: PlantCARE (Lescot *et al.*, 2002) and PLACE (Higo *et al.*, 1999). The AGRIS information server was also searched for *At2g02360* promoter-related data (Yilmaz *et al.*, 2011). A transcriptome analysis was performed using a protein BLAST search in the translated EST databases (TBLASTN) from Arabidopsis. Large scale expression data sets and microarray data as available on the eFP browser (Winter et al. 2007) were analyzed to obtain a hypothetical expression dataset for the *At2g02360* gene in Arabidopsis cells and tissues. To retrieve genes co-expressed with the *At2g02360* gene, co-expression meta-analyses were performed using Genevestigator (Hruz *et al.*, 2008), ATTED-II (Obayashi *et al.,* 2011), Expression Angler (Toufighi *et al.*, 2005) and the CSB.DB co-response database (Steinhauser *et al.,* 2004). The reliability of co-

expression was scored by the databases based on a Pearson's correlation coefficient in the range of 0-1. Genes with a co-expression correlation score above the cut-off of 0.5 were listed and organized with a Venn diagram to present the genes retrieved from more than one database as shared elements.

3.3.11 Development of an anti-F-box-Nictaba antibody

A peptide-based antibody specific against the F-box-Nictaba protein has been produced in a guinea pig production platform by the Thermo Scientific custom antibody service. Based on the analysis of the AA sequence of the F-box-Nictaba protein with the Antigen Profiler software of Thermo Scientific (http://www.pierce-antibodies.com/custom-antibodies/peptide-design-antigen-profiler.cfm) the peptide (^{201}CFSEAIRRGRRNVVKPKQRE^{220}) was selected for immunization. Before and after immunization, sera of selected animals were tested by Western blot for reactivity against purified (recombinant) F-box-Nictaba and its Nictaba domain (described in Chapter 2) as well as against total protein extracts from WT A. thaliana plants and WT Arabidopsis PSB-D cells (*i.e.* a dark-grown cell line from the VIB Department of Plant Systems Biology, Ghent University).

3.3.12 Protein extraction

The protein extracts used to test the anti-F-box-Nictaba antibody were extracted using 20 mM 1,3-diaminopropane. The total protein extracts used to analyse transgenic OE lines were extracted with 1xPBS containing a mix of protease inhibitors including 1mM PMSF and 1 µg/ml of pepstatin, leupeptin and aprotinin (all from Sigma). All extractions were performed by grinding the material with a mortar and pestle in the presence of the corresponding extraction solution. The resulting crude protein extracts were transferred to 1.5 ml eppendorf tubes, centrifuged at 4°C for 10 min at 12,000 rpm. Supernatants were collected for subsequent SDS-PAGE and Western blot analysis.

3.3.13 SDS-PAGE and Western blot analysis

SDS-PAGE and Western blot analyses were performed as described in Chapter 2, Section 2.3 with modifications as follows. Western blot analysis for prescreening of guinea pig sera was performed using 1/10 diluted sera as primary antibodies and a HRP-coupled goat anti-guinea pig IgG antibody (1/10,000) (Invitrogen) as secondary antibody. For the final antibody test, the anti-F-box-Nictaba serum (1/500 diluted) was used as primary antibody, together with a HRP-coupled goat anti-guinea pig IgG antibody (1/10,000) (Invitrogen) as secondary antibody, followed by an additional 1h incubation with PAP (peroxidase-anti-peroxidase) (1/2,000) (Sigma).

3.3.14 Phenotypic analysis of transgenic *A. thaliana* plants

3.3.14.1 Germination rate assessment

For the germination assay, two hundred surface sterilized seeds of WT *A. thaliana* plants and of transgenic KO plants (impaired in *At2g02360* gene expression) and OE plants (overexpressing the *F-box-Nictaba* gene *At2g02360*) were directly sown on MS agar plates (25 seeds/line/plate). After three days of stratification, plates were transferred to a controlled growth chamber set at 21°C with a 16/8 h light/dark photoperiod and germinated seeds were counted daily for five days.

3.3.14.2 Leaf size analysis

Approximately hundred fully expanded rosette leaves collected from 5-week-old WT *A. thaliana* plants as well as from transgenic *F-box-Nictaba*-specific KO and OE plants were scanned with a flatbed scanner (Canoscan Lide 25, Canon, Diegem, Belgium) at 1200 dpi. Scans of single leaves were processed with the ImageJ software package. The leaf area was measured with the APS Assess 2.0 program (Lamari, 2008) using 1200 dpi calibration.

3.3.14.3 Thermotolerance assessment

Thermotolerance experiments were performed according to the protocol adapted from Chae *et al.* (2013) and Li *et al.* (2013). About fifty seeds of WT *A. thaliana* plants as well as of transgenic *F-box-Nictaba*-specific KO and OE plants were directly sown on MS agar plates (25 seeds/line/plate). After 3 days of stratification, the plants were transferred to a controlled growth chamber set at 21°C with a 16/8 h light/dark photoperiod for one week. Then, 7-day-old seedlings were incubated at different temperatures for the assessment of heat stress tolerance. For basal thermotolerance, plants were treated with 45°C for 1h and put back to 21°C for recovery. For acquired thermotolerance, plants were first pre-treated with 37°C for 1h and then allowed to recover at 21°C for 3h before final treatment with the heat stress at 45°C for 1h, after which plants were again transferred to 21°C. At indicated time points, samples were taken for gene expression analysis by qRT-PCR. Survival rates were calculated six days after treatments, where seedlings showing obvious etiolation appearance were considered dead.

3.3.14.4 Response to *Pseudmonas syringae* infection

Approximately sixty individually grown 5-week-old WT *A. thaliana* plants as well as transgenic *F-box-Nictaba*-specific KO and OE plants were inoculated with either the *Pst* DC3000 infection or the mock solutions as described above. At 0, 3 and 4 days post infection (dpi) approximately hundred leaves were collected for each line and were scanned with a flatbed scanner (Canoscan Lide 25) at 1200 dpi. Scans of single leaves were processed with the ImageJ software package. Leaf damage was

determined as % lesion of the total leaf and was quantified using the disease assessment software of the APS Assess 2.0 program (Lamari, 2008).

3.4 Results

3.4.1 *In silico* expression analysis indicates that *At2g02360* is a stress-responsive gene

To obtain information related to the gene regulatory elements in the promoter sequence of the F-box-Nictaba encoding gene *At2g02360* in Arabidopsis, an *in silico* survey was performed using the PLACE, PlantCARE and AGRIS databases which contain both experimentally validated and predicted information about upstream regions of annotated Arabidopsis genes including *cis*-regulatory elements and transcription factor binding sites. The 1800 nt upstream region of the *At2g02360* gene was used as the input sequence (see supplementary Fig. A3.1). The regular core promoter elements, *i.e.* the CAAT box, CCAAT box and TATA box, were found 15, 3 and 3 times respectively, indicating that the sequence encodes a promoter sequence.

Analysis of this promoter sequence for tissue or stress-related elements yielded a list of many putative *cis*-regulatory elements related to responsiveness towards hormonal stress as well as abiotic stress signaling. According to both the PLACE and PlantCARE databases, which gather resources for different vascular plants, light-responsive elements such as GATA boxes, CT1CONSENSUS and I boxes constituted the largest group, followed by putative drought stress-responsive elements such as MYB1AT and MYCCONSENSUSAT, a salt-responsive element and many hormone-responsive elements, including elements responsive towards auxin, ABA, SA, GA and ET. In contrast, the survey of Arabidopsis-specific AGRIS database resulted in a much shorter list of putative *cis*-elements including only the SA-responsive W-box element (binding site for WRKY transcription factors), an ABA-responsive DPBF1&2 binding site, a MYB4 binding site (indicative for environmental stress response) and a LFY consensus site. A complete overview of all putative *cis*-regulatory elements identified by PLACE, PlantCARE and AGRIS can be found in supplementary Table A3.4. Interestingly, the *F-box-Nictaba* promoter sequence was found to also contain some putative *cis*-acting elements which are associated with gene-specific expression in trichomes (supplementary Fig. A3.3).

A transcriptome analysis by TBLASTN searches against the Arabidopsis EST database indicated expression of *At2g02360* upon various hormone and abiotic stress treatments (Results not shown). The eFP browser comprises gene expression data mostly gathered from ATH1 affymetrix microarray and genome tilling array experiments. Screening of this data set revealed that during normal development of *A. thaliana At2g02360* was seemingly more expressed in rosette and cauline leaves compared to other tissues of the plant. Upon heat stress (38°C) and SA treatment (10 µM) the *At2g02360* gene was up-regulated by 2-fold in all plant tissues. Similarly, mannitol (300 mM) treatment resulted in 2-fold elevated *At2g02360* expression levels, more specifically in the leaves.

Infection with a virulent *Pseudomonas* species also enhanced *At2g02360* expression. In contrast, *At2g02360* expression was two times down-regulated after salt treatment. However, before drawing conclusions this putative stress-regulated expression of *At2g02360* needs to be confirmed by a functional analysis.

3.4.2 Expression of the *F*-box-*Nictaba* gene throughout the development of WT *A. thaliana* plants

Since the *A. thaliana* genome encodes multiple *F-box-Nictaba* related genes with similar DNA sequences, primers to perform qRT-PCR were designed to bind to the most distinct fragments of the *At2g02360* coding sequence (most different from other F-box-Nictaba homologs in *A. thaliana*). Out of two primer pairs tested only the combination evd786–evd787 (supplementary Table A3.1) yielded a single PCR amplification fragment after electrophoresis on agarose gel. Sequencing of this fragment returned exclusively the *At2g02360* sequence confirming that the primers are specific for the gene of interest.

To determine the expression pattern for *F-box-Nictaba* in WT *A. thaliana* plants during normal development, RNA samples were extracted from plant material collected at different developmental stages originating from plants grown under standard conditions and used in qRT-PCR analyses. As illustrated in Fig. 3.1A, *At2g02360* was expressed during all developmental stages and in every tissue tested. Only in roots from young plants and in flowers, there was a slightly lower expression of *At2g02360* compared to the youngest stage tested (*i.e.* cotyledons stage).

3.4.3 *F-box*-Nictaba gene expression is up-regulated after SA application and heat shock treatment

In order to evaluate the experimental setup and successful induction of plant stress specific responses, positive control genes were included for all hormone and stress treatments (supplementary Table A3.1). These genes have previously been experimentally demonstrated as hormone- or stress-inducible (according to Goda *et al.*, 2008; Kim *et al.*, 2010b; Nishizawa-Yokoi *et al.*, 2010; Besseau *etal.,* 2012; Espunya *et al.*, 2012). In this study, all positive control genes were significantly up-regulated after application of different hormones and stresses (Results not shown).

The uniform expression profile of *F-box-Nictaba* gene observed throughout plant development (Fig. 3.1A) changed considerably when Arabidopsis plants were subjected to exogenous stress treatments. After 1 hour of SA treatment, *At2g02360* expression was slightly down-regulated compared to the mock treatment, but *At2g02360* mRNA levels were significantly up-regulated after 3 h of SA application with a maximal increase in expression level of almost 4-fold after 10 h of SA application. Transcript levels in treated plants slightly dropped after 24 h but were still 2-fold higher than in the untreated plants. Transcript levels for the SA-inducible transcription factor WRKY70 were up-

regulated faster and reached much higher levels compared to *F-box-Nictaba* expression levels (Fig. 3.1B).

Also heat stress enhanced *F-box-Nictaba* gene expression with a maximal up-regulation of almost 3-fold after 10 h of heat stress compared to control plants. However, the expression of the positive control gene *Hsp70b*, encoding a heat shock-responsive chaperone protein, was enhanced much faster and reached much higher mRNA levels compared to those measured for *At2g02360* (Fig. 3.1C).

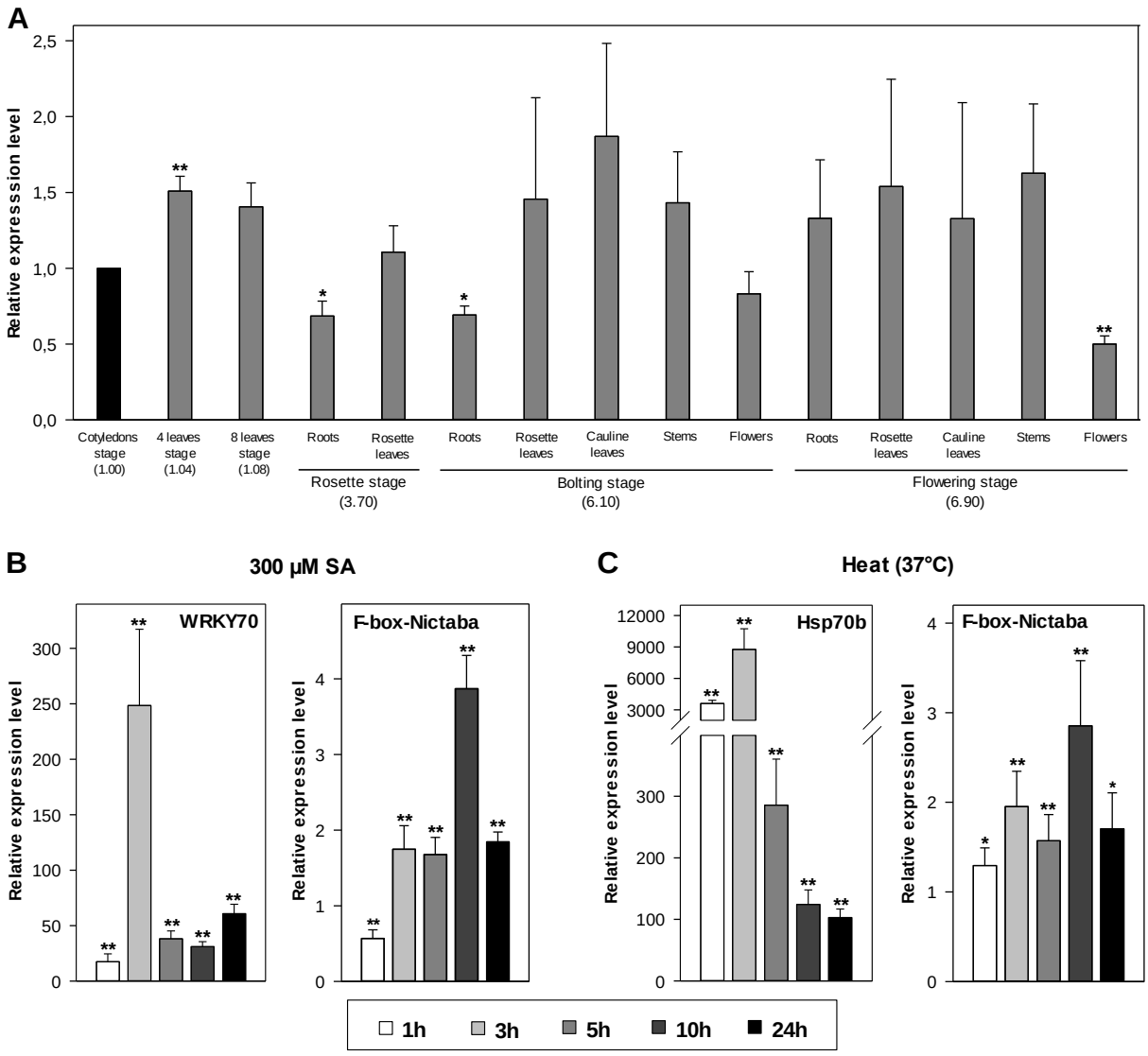


Fig. 3.1 Relative transcript levels of *At2g02360* in WT *A. thaliana* Col-0 plants determined by qRT-PCR analyses of two independent biological experiments. *n*=2; error bars ± SE. Asterisks indicate statistically significant differential expression compared to control samples (*p<0.05; **p<0.01). A, *At2g02360* expression levels measured at different developmental plant stages (as presented in Fig. 3.3 and according to Boyes *et al.*, 2001) and in different organs of Arabidopsis plants grown under standard conditions. Expression levels are presented relatively compared to the *At2g02360* expression level determined in the cotyledons stage. B, *At2g02360* and *At3g56400* (encoding WRKY70) expression levels in 16-day-old Arabidopsis seedlings after 300 µM SA treatment, presented relatively compared to gene expression levels determined in the mock-treated plants. C, *At2g02360* and *At1g16030* (encoding Hsp70b) expression levels in 16-day-old Arabidopsis seedlings after a 37°C heat shock treatment, presented relatively compared to gene expression levels determined in the mock-treated plants.

Other hormones (ABA, GA_3, BAP) and abiotic stress treatments (NaCl and proteasome inhibitor MG132) either did not affect *F-box-Nictaba* gene expression at all, down-regulated it slightly as was the case for treatments with IAA, MeJA and cold, or resulted in an early and very low (1,5-fold) up-regulation (mannitol) (supplementary Fig. A3.2). As for the ethephon treatment, *F-box-Nictaba* was first slightly down-regulated, but then increased 2-fold after 10 h of treatment. In view of this apparent up-regulation, an additional later time point (24 h) was also analyzed but did not reveal any differential expression for this treatment. The experiment was performed again by treatment of plantlets with a higher ethephon concentration (300 µM), but also here, no differential expression of the *F-box-Nictaba* gene was detected for any of the tested time points (Results not shown).

3.4.4 *F*-box-*Nictaba* expression is up-regulated in WT *A. thaliana* plants after *Pseudomonas* infection but is slightly down-regulated by fungal infection

To investigate *At2g02360* expression after biotic stress application, 5-week-old WT *A. thaliana* Col-0 plants were infected with the virulent hemibiotrophic bacterium *P. syringae* pv. *tomato* strain DC3000 (*Pst* DC3000) and with the necrotrophic fungus *B. cinerea* strain B05.10. mRNAs sampled from infected rosette leaves were analyzed for *At2g02360* transcript levels in qRT-PCR assays.

As illustrated in Fig. 3.2A-C, bacterial infection of WT plants with *Pst* DC3000 strongly enhanced the expression of both control genes *WRKY70* (Fig. 3.2A) and *PR1* (Fig. 3.2B) with 14- and 18-fold at 1 and 2 dpi, and up to over 1500-fold at 3 dpi, respectively, compared to mock-sprayed plants. The *F-box-Nictaba* mRNA levels were significantly up-regulated approximately 2.5-fold at 1, 2 and 7 dpi. The expression dropped at 3 and 5 dpi but was still higher than in the mock-treated plants (Fig. 3.2C).

Fungal infection of WT plants with *B. cinerea* mildly affected *F-box-Nictaba* mRNA levels compared to mock-treated plants (Fig. 3.2F). At 2 and 3 dpi, *At2g02360* expression levels were almost 2-fold reduced. The expression of the control gene *PR1* was not significantly altered upon *Botrytis* infection apart from very small down-regulation at 4 dpi (Fig. 3.2E). In contrast, the expression of the control gene *PDF1.2* was highly up-regulated after *Botrytis* infection reaching a maximum of 50 times increase in infected tissues at 3 dpi (Fig. 3.2D).

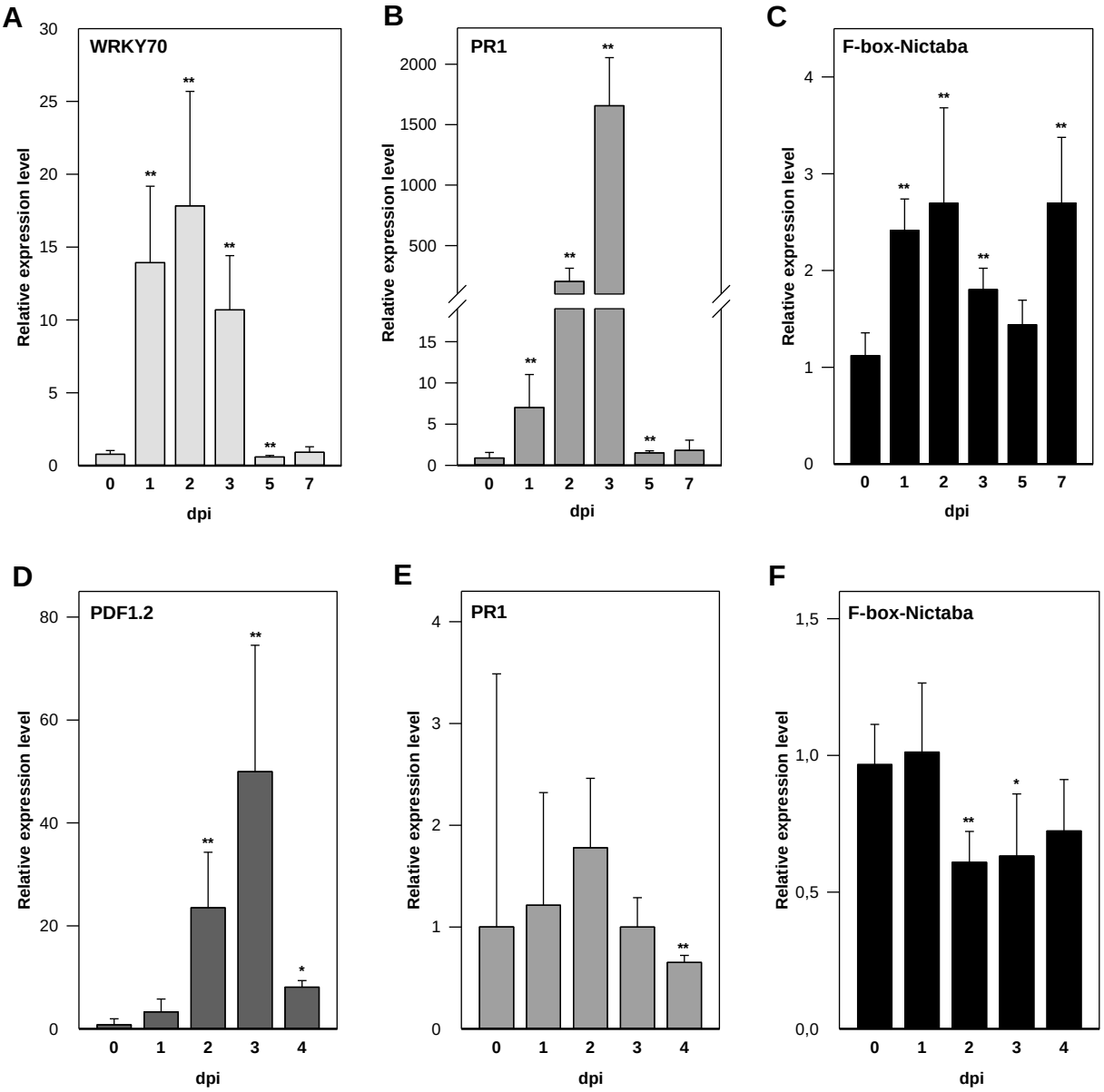


Fig. 3.2 Relative transcript levels of *At2g02360* and selected positive control genes determined by qRT-PCR in 5-week-old WT *A. thaliana* Col-0 plants after pathogenic infection. Expression levels are presented relatively compared to the gene expression levels determined in the mock-treated plants. Values were obtained by two independent biological replicates. *n*=2; error bars ± SE. Asterisks indicate statistically significant differential expression compared to mock-treated plants (*p<0.05; **p<0.01). A-C, Relative expression of *At3g56400* (encoding WRKY70) (A), *At2g14610* (encoding PR1) (B) and *At2g02360* (encoding F-box-Nictaba) (C) after infection of WT Arabidopsis plants with *Pst* DC3000. D-F, Relative expression of *At5g44420* (encoding PDF1.2) (D), *At2g14610* (encoding PR1) (E) and *At2g02360* (F) after infection of WT Arabidopsis plants with *B. cinerea* strain B05.10.

3.4.5 The *F-box-Nictaba* promoter is particularly active in leaf trichomes

To test the activity of the 5'-upstream region of the *At2g02360* gene, a promoter *At2g02360*:GUS reporter construct was created and introduced into *A. thaliana* plants of Col-0 background. Three independent lines were isolated and used in histochemical assays. In a parallel experiment, a CaMV35S:GUS line was used as the positive control. Preliminary experiments performed on 14-day-old *pAt2g02360:GUS* seedlings revealed a distinct and localized GUS staining in the leaf trichomes (Fig. 3.3A). Subsequently, plants grown under standard conditions were also analyzed at different developmental stages as depicted in Fig. 3.3B. Overall, the *pAt2g02360:GUS* plants showed comparable GUS staining patterns for different transgenic lines, but the intensity of the GUS staining differed amongst plants (Fig. 3.3C). In the very young seedlings (stage 0.7) no GUS staining was detected in any of the tested plants. In the cotyledons and 2 leaves stage (stages 1.00 and 1.02), plantlets showed a first weak GUS activity spread over the mesophyll cells but absent from the shoot meristem, the hypocotyl or the root system. When plants developed (from stage 1.08 onwards), a very intense GUS activity was observed all over the petioles and in the majority of the trichomes present on the shoot meristem and first leaves. The very specific, localized GUS staining in the trichomes present on newly developing leaves remained throughout further development of the plants. In plantlets of stage 1.10 and older (i.e. rosette stage), GUS staining was prominently visible in trichomes present on new leaves and in the petioles, the major leaf vein and some parts with mesophyll cells of older leaves. Trichomes present on older leaves also showed GUS staining, especially in the trichomes located in close proximity of the petiole, but their GUS staining was less intense compared to the staining detected in trichomes residing on young leaves. In flowering plants (stage 6.90), GUS staining was mainly visible in the flowers, some siliques and in some major veins and trichomes located in close proximity of these veins on rosette and cauline leaves. The CaMV35S:GUS control plants showed an intense blue, homogeneous GUS staining pattern in all organs and tissues of the plants, throughout all developmental stages tested (results not shown).

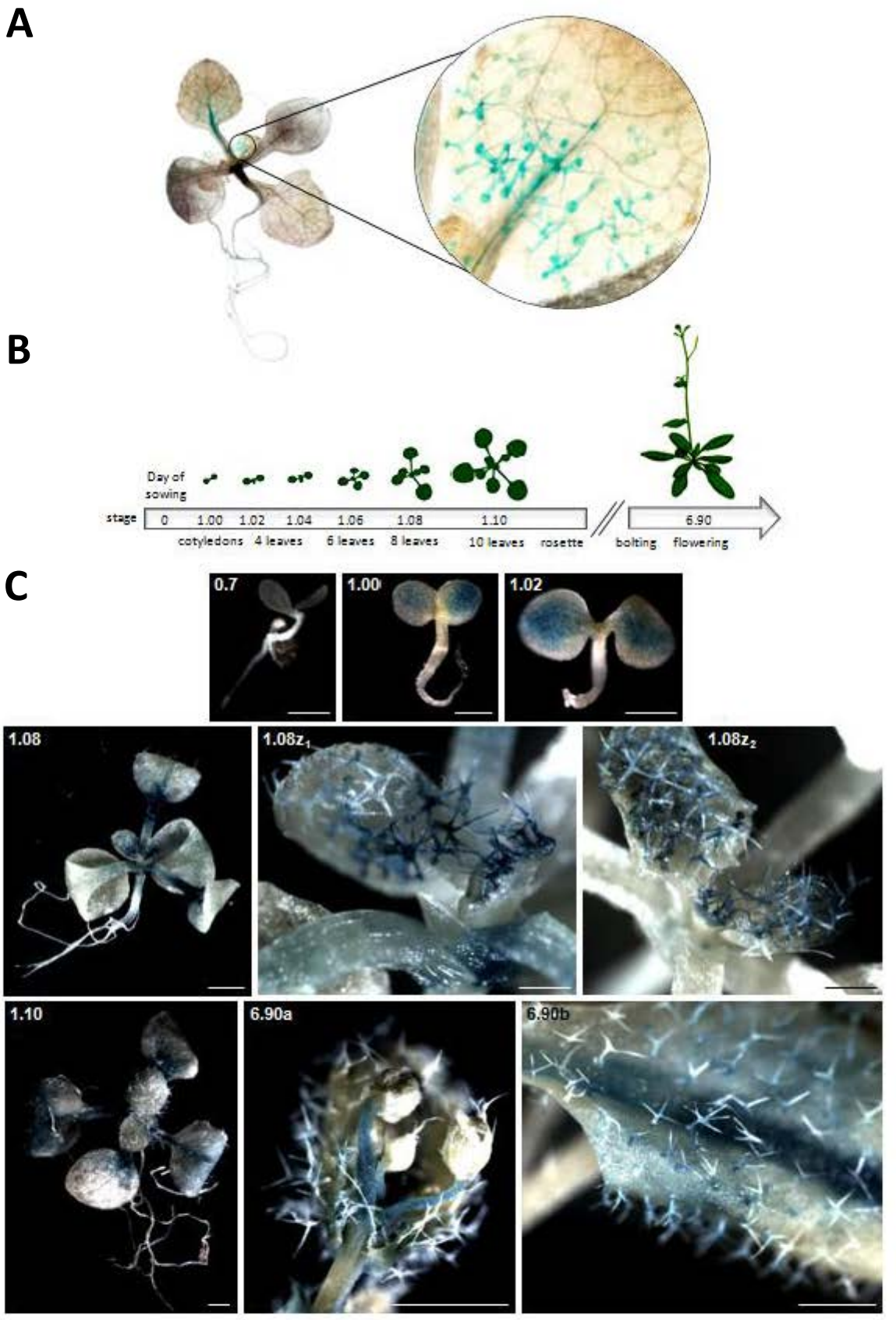


Fig. 3.3 Histochemical analysis of *pAt2g02360:GUS* Arabidopsis lines. A, Preliminary GUS assay result performed on 14-day-old plantlets. B, Different developmental plant stages analyzed throughout the assays (according to Boyes *et al.*, 2001). C, GUS staining data for selected developmental stages. Panels $1.08z_1$ and $1.08z_2$ represent a closer look of trichomes present on new leaves; panel 6.90a shows a closer look of trichomes present on flower buds; panel 6.90b represents a close-up of rosette leaves. Scale bars represent 1 mm.

3.4.6 *At2g02360* is co-expressed in a network of defense-related genes

To predict the putative function of *At2g02360* and to identify other Arabidopsis genes which are functionally related, the gene co-expression network for *At2g02360* was built using the meta-analysis databases Genevestigator, ATTED-II, Expression Angler and the CSB.DB co-response database (Fig. 3.4 and supplementary Table 3.1). This search revealed that one third of the retrieved co-expressed genes are involved in plant defense responses against pathogens. More than half of these defense-related genes was revealed by at least two of the four screened databases. Two of them, *At3g28940* and *At3g28950* (both encoding AIG2 (avrRpt2-induced gene 2)-like protein) are co-expressed according to all four databases and showed a high co-expression correlation score (>0.70). Furthermore, *At1g64280* (encoding regulatory protein NPR1) retrieved by ATTED-II and CSB.DB, as well as *At4g19660* (encoding regulatory protein NPR4) shown by ATTED-II and Genevestigator, are key regulators of plant responses against bacterial pathogens and their function relies on or is modulated by the UPS (Fu *et al.,* 2012; Spoel *et al.,* 2009). Two resources (ATTED-II and CSB.DB) also returned a gene encoding WRKY25 (*At2g30250*), a stress-induced transcription factor recognizing the W-box sequences functioning as a negative regulator of SA-mediated defense responses to *P. syringae* (Zheng *et al.,* 2007).

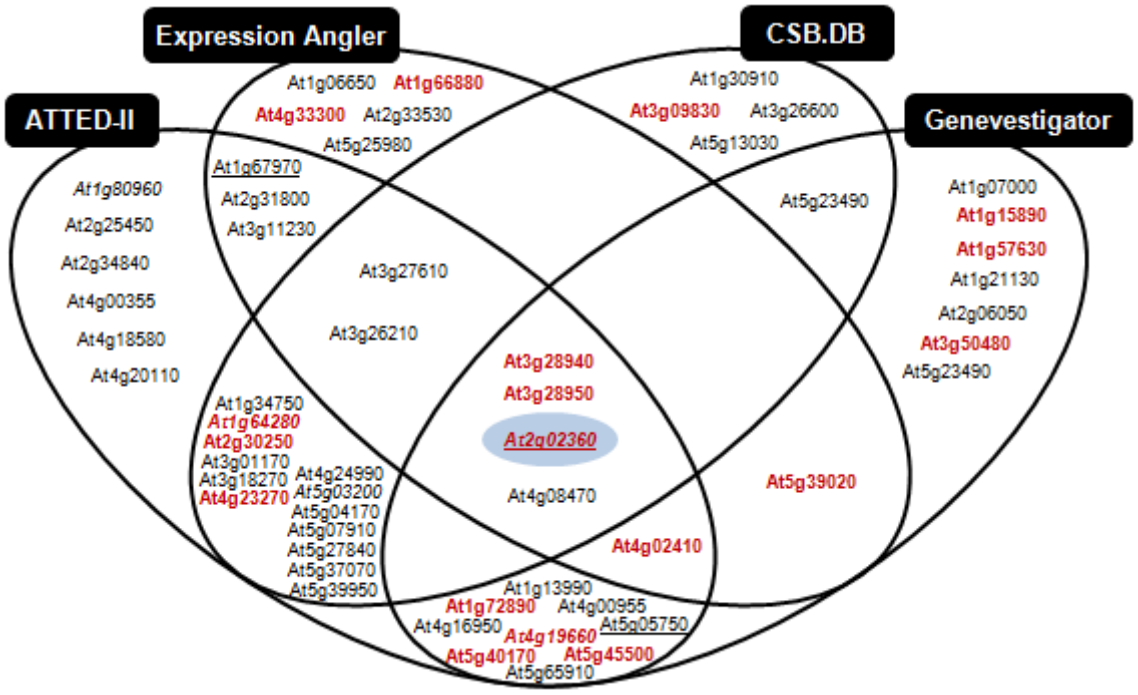


Fig. 3.4 Gene co-expression network for the F-box-Nictaba gene (*At2g02360*) defined by a meta-analysis databases search using Genevestigator, ATTED-II, Expression Angler and the CSB.DB co-response database. Scoring systems used by different databases to determine the reliability of co-expression rely on a Pearson's correlation coefficient in the range of 0-1. Coefficient of '1' indicates a strong relationship for gene expression regulation and '0' indicates no relationship. Genes with co-expression correlation score above the cut-off of 0.5 were retrieved. Genes indicated by more than one tool are presented as shared elements. **Genes related to defense responses against pathogens are shown in red and in bold**. Genes related to heat stress responses are underlined. *Genes involved in the UPS are shown in italics.*

Table 3.1 Genes co-expressed with *At2g02360* defined by a meta-analysis databases search using Genevestigator, ATTED-II, Expression Angler and the CSB.DB co-response database.

Gene ID	Gene name	Co-expression score
At1g06650	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	0.72
At1g07000	Exocyst subunit exo70 family protein B2	0.51
At1g13990	Uncharacterized protein	0.51
At1g15890	**Probable disease resistance protein At1g15890**	**0.55**
At1g21130	Indole glucosinolate *O*-methyltransferase 4	0.52
At1g30910	Molybdenum cofactor sulfurase-like protein	0.67
At1g34750	Probable protein phosphatase 2C 10	0.59
At1g55450	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	0.68
At1g57630	**Disease resistance protein RPP1-WsB**	**0.51**
At1g64280	***Regulatory protein NPR1***	***0.58***
At1g66880	**Serine/threonine protein kinase**	**0.73**
At1g67970	Heat stress transcription factor A-8	0.71
At1g72890	**TIR-NBS class of disease resistance protein**	**0.55**
At1g80960	*F-box protein At1g80960*	*0.53*
At2g06050	12-oxophytodienoate reductase 3	0.51
At2g25450	Probable 2-oxoacid dependent dioxygenase	0.52
At2g30250	**Probable WRKY transcription factor 25**	**0.58**
At2g31800	Integrin-linked protein kinase family protein	0.69
At2g33530	Serine carboxypeptidase-like 46	0.70
At2g34840	Coatomer subunit epsilon-2	0.52
At3g01170	Ribosomal protein L34e superfamily protein	0.57
At3g09830	**Protein kinase family protein**	**0.65**
At3g11230	Yippee family putative zinc-binding protein	0.74
At3g18270	Uncharacterized protein	0.56
At3g26210	Cytochrome P450 71B23	0.69
At3g26600	Armadillo repeat only 4 protein	0.69
At3g27610	Nucleotidylyl transferase domain-containing protein	0.73
At3g28940	**AIG2(avrRpt2-induced gene 2)-like protein**	**0.71**
At3g28950	**AIG2(avrRpt2-induced gene 2)-like protein**	**0.71**
At3g50480	**RPW8-like protein 4**	**0.56**
At4g00355	Uncharacterized protein	0.55
At4g00955	Uncharacterized protein	0.50
At4g02410	**L-type lectin (Concanavalin A)-domain containing receptor kinase IV.3**	**0.66**
At4g08470	Putative mitogen-activated protein kinase MEKK3	0.79
At4g16950	Uncharacterized protein	0.53
At4g18580	Uncharacterized protein	0.52
At4g19660	***Regulatory protein NPR4***	***0.51***
At4g20110	Vacuolar-sorting receptor 7	0.53
At4g23270	**Cysteine-rich receptor-like protein kinase 19**	**0.66**
At4g24990	Membrane-anchored ubiquitin-fold protein 3	0.55
At4g33300	**Probable disease resistance protein At4g33300**	**0.71**
At5g03200	*Protein LOG2-LIKE UBIQUITIN LIGASE 1*	*0.54*
At5g04170	Calmodulin-like protein 50	0.54
At5g05750	DNAJ heat shock N-terminal domain-containing protein	0.53
At5g07910	Uncharacterized protein	0.54
At5g13030	Uncharacterized protein	0.64
At5g23490	Uncharacterized protein	0.56
At5g25980	Myrosinase 2	0.73
At5g27840	Serine/threonine-protein phosphatase PP1 isozyme 8	0.56
At5g37070	Uncharacterized protein	0.65
At5g39020	**Malectin/receptor-like protein kinase family protein**	**0.68**
At5g39950	Thioredoxin H2	0.61
At5g40170	**Receptor like protein 54**	**0.50**
At5g45500	**RNI-like superfamily protein**	**0.53**
At5g65910	BSD domain-containing protein	0.51

For each gene its co-expression correlation score with *At2g02360* is shown. In case of genes indicated by more than one tool, the highest correlation score is shown.

Red and in bold: genes related to defense responses against pathogens

Underlined: genes related to heat stress responses

In italics: genes involved in the UPS

Two other genes co-expressed with *F-box-Nictaba* are related to the UPS: the gene encoding the F-box protein At1g80960 (according to ATTED-II) and the *At5g03200* gene encoding the LOG2-like Ub ligase 1 (ATTED-II and CSB.DB). Finally, two genes encode heat stress-related proteins: At5g05750 (revealed by ATTED-II and Genevestigator) - a DNAJ heat shock N-terminal domain-containing protein, and At1g67970 (ATTED-II and Expression Angler) - a heat stress transcription factor A-8. One of the genes, *At4g08470*, is also co-expressed according to all four databases (next to the two genes encoding AIG2-like proteins) and showed a very high co-expression correlation score (>0.70). It encodes a putative mitogen-activated protein kinase MEKK3, thus presumably functions in protein phosphorylation.

3.4.7 Phenotypic analysis of transgenic *A. thaliana* plants with altered F-box-Nictaba expression.

Since *F-box-Nictaba* gene expression was significantly up-regulated after SA and infection with *Pst* DC3000 as well as after heat stress application, it was suggested that F-box-Nictaba plays a role in plant defense responses. Thus, to investigate the relevance of F-box-Nictaba for plant resistance towards stress, it was checked whether altered F-box-Nictaba expression would influence the resistance of Arabidopsis plants to selected stress treatments. For this purpose transgenic Arabidopsis plants were used, which are impaired in *F-box-Nictaba* gene expression (KO lines) as well as lines which overexpress *F-box-Nictaba* (OE lines).

3.4.7.1 Validation of the F-box-Nictaba-specific antibody

In order to confirm the overexpression of F-box-Nictaba in the transgenic *A. thaliana* lines, a specific antibody was required which could distinguish the F-box-Nictaba protein encoded by *At2g02360* from the other highly homologous F-box proteins with a Nictaba domain (for overview see Delporte *et al.,* 2015). To avoid aspecific reactivity, an antibody against F-box-Nictaba has been developed based on a 20 AA peptide (^{201}CFSEAIRRGRRNVVKPKQRE^{220}) present in the most distinct region of the Nictaba domain of the F-box-Nictaba sequence.

Out of two selected immunized guinea pigs, the serum of only one of the animals was reactive against the purified F-box-Nictaba protein and Nictaba domain (Fig. 3.5, lanes 1 and 2) without detecting the tobacco lectin Nictaba (Fig. 3.5, lane 5) and producing only a moderate background in the lanes containing total protein extracts from WT Arabidopsis plants and cells (Fig. 3.5, lanes 3 and 4). The negative control, where serum before immunization was used as primary antibody, returned no signal at all with little background (result not shown). The peptide-based antibody should therefore be specifically directed against the F-box-Nictaba protein from *A. thaliana*.

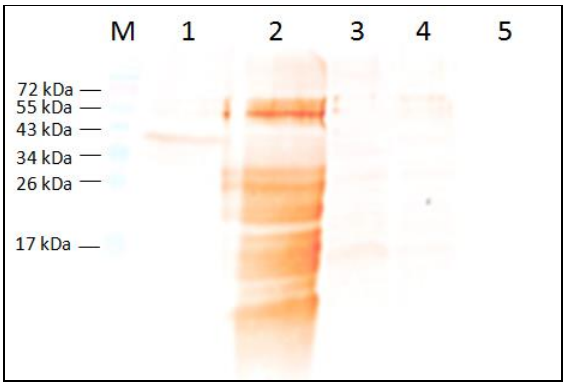


Fig. 3.5 Specificity test of the anti-F-box-Nictaba antibody on different purified proteins and total protein extracts from WT *A. thaliana* plants and cells by Western blot using the serum of immunized animal PY0147 (1/500 dilution) as primary antibody. M: protein marker; Lane 1: purified recombinant F-box-Nictaba (1 µg); Lane 2: purified recombinant Nictaba domain of F-box-Nictaba (1 µg); Lane 3: total protein extract from WT *A. thaliana* plants (50 µg); Lane 4: total protein extract from WT *A. thaliana* cells (50 µg); Lane 5: purified recombinant Nictaba from tobacco (1 µg).

3.4.7.2 Selection of transgenic KO and OE lines

Line SALK_085735C (KO6) is a true KO mutant impaired in *F-box-Nictaba* gene expression

The SALK population comprises single, segregating flank-tagged T-DNA insertion lines generated by Dr. Joseph Ecker (The Salk Institute in California, USA) via *A. tumefaciens* vacuum infiltration of Arabidopsis ecotype Col-0 (Alonso *et al.,* 2003). In the past decade, these mutant lines have been widely exploited for functional genetic studies in *A. thaliana* (Ulker *et al.,* 2008).
Based on the information retrieved from the SALK database, two SALK T-DNA lines have been selected with a T-DNA insertion in exon sequences of *At2g02360*, i.e. in SALK_007866 (KO4) and SALK_085735C (KO6) (Fig. 3.6A).

KO4 and KO6 plants were tested for homozygosity by PCR on total genomic DNA using the left border primer of the T-DNA insertion (LBb1.3) as well as the line-specific left (LP) and right (RP) genomic primers spanning the insertion site (supplementary Table A3.2; Fig. 3.6B-C). By using gene-specific primers spanning the insertion site (primer combinations 1 and 3), PCR products of 1067 bp and 1060 bp expected for WT allele were amplified from WT DNA but not from mutant DNA. PCRs with the T-DNA-specific primer and RP gene-specific primers (primer combinations 2 and 4) showed products in the range of 700 bp for mutant DNAs, but not for the WT line (Fig. 3.6B). This is in good agreement with the expected products for mutant alleles of 795 bp and 746 bp for KO4 and KO6, respectively. Thus, both tested SALK T-DNA insertion lines are homozygous.

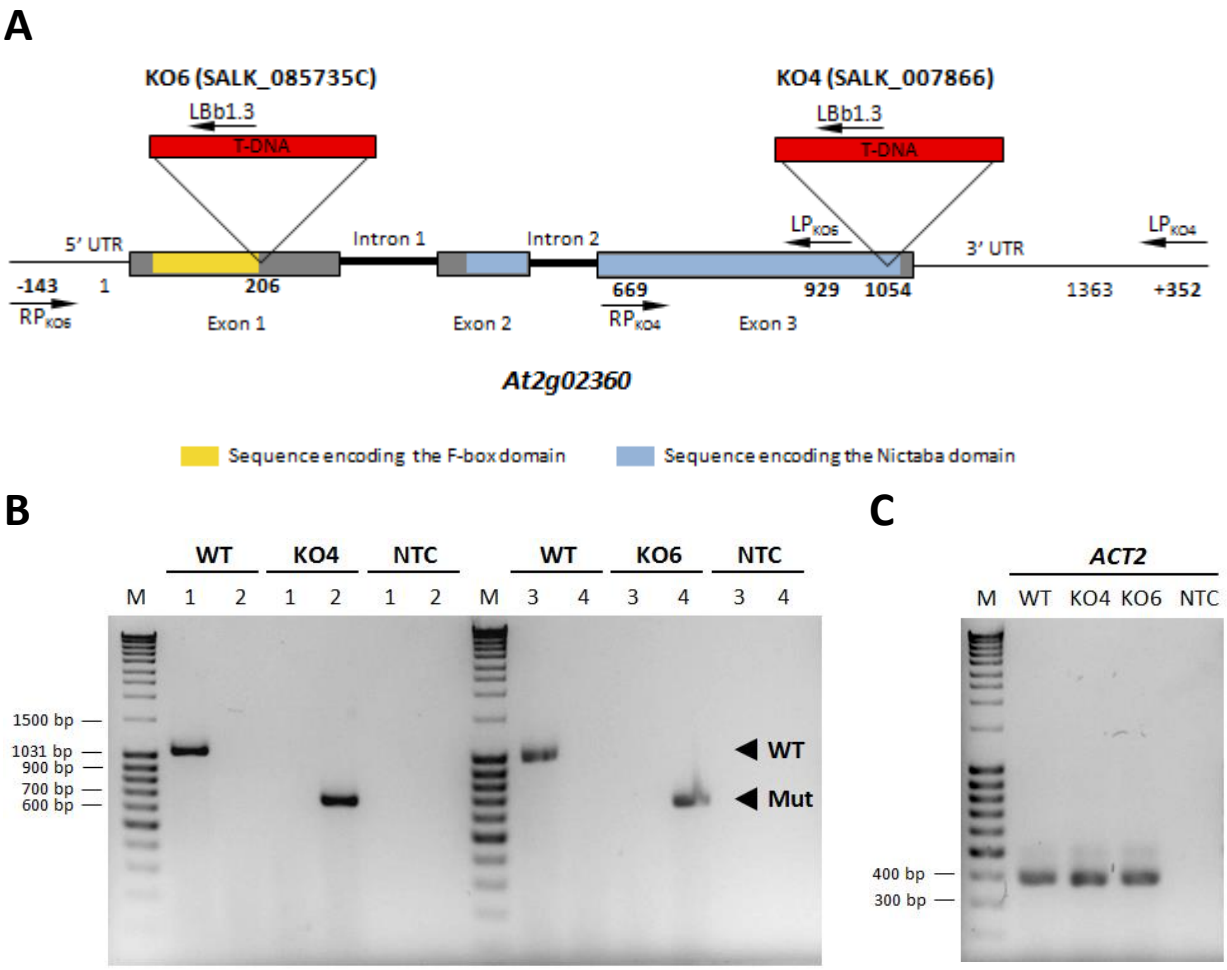


Fig. 3.6 Selection of transgenic KO lines. A, Genomic organization of the *F-box-Nictaba* gene *(At2g02360)* (1-1363 bp) with indicated sites of T-DNA insertions within the gene as in the mutant lines KO4 (SALK_007866) and KO6 (SALK_085735C). Binding positions for primers used for testing of mutant lines are marked with arrows and corresponding primer names. B, PCR on genomic DNA of WT plants and mutant T-DNA insertion lines KO4 and KO6 demonstrating homozygosity of the transgenic lines. Lanes 1: Primer combination LP_{KO4}+RP_{KO4}; Lanes 2: Primer combination LBb1.3 + RP_{KO4}; Lanes 3: Primer combination LP_{KO6}+RP_{KO6}; Lanes 4: Primer combination LBb1.3 + RP_{KO6}. WT: WT allele, Mut: mutant allele. C, DNA quality tested with actin (*ACT2*) primers. M: DNA marker; NTC: no template control.

Next, RT-PCR and qRT-PCR were performed to analyze whether the *F-box-Nictaba* transcript is indeed absent in the two presumed KO lines. As presented in Fig. 3.7A, the full-length F-box-Nictaba sequence (819 bp) could be amplified both in cDNA of WT plants and of KO4 plants, but not in cDNA samples of KO6 plants. Fig. 3.7B clearly demonstrates that *F-box-Nictaba* transcript levels in the KO4 line are indeed not reduced but are 3-fold higher than in the WT plants. In contrast to line KO4, *F-box-Nictaba* expression in the KO6 mutant line is over 20-fold lower than in the WT plants. Altogether, these data demonstrate that the KO6 line is a true KO line and that despite T-DNA insertion, *F-box-Nictaba* is still expressed in the KO4 plants. Most probably, the T-DNA sequence present in the *At2g02360* sequence in the KO4 line was not inserted at the predicted insertion point, but is rather introduced at the 3'UTR of the gene where it does not prevent *At2g02360* from transcript expression. What is more, since the KO4 plants present an even higher *F-box-Nictaba*

expression than the WT plants it could be hypothesized that the T-DNA insertion occurred at a site of a negative regulatory sequence of the *At2g02360* gene. Therefore, these KO4 plants were excluded from subsequent plant phenotypic and physiological assays.

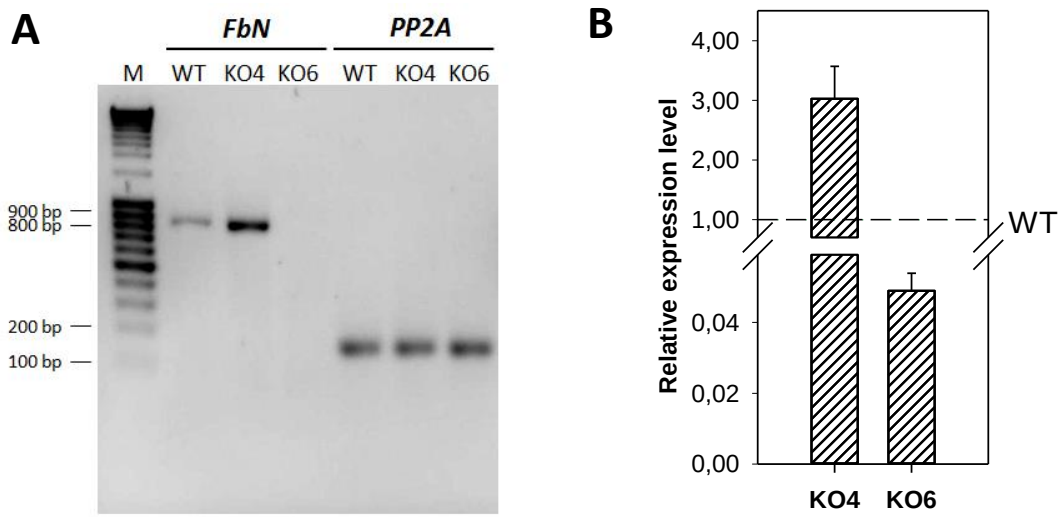


Fig. 3.7 Expression analysis of *F-box-Nictaba* in putative KO lines. A, RT-PCR on cDNA isolated from 3-week-old WT plants and from mutant T-DNA insertion lines KO4 and KO6. M: DNA marker; *FbN: F-box-Nictaba* gene*; PP2A: protein phospatase 2* reference gene. B, Relative expression of *F-box-Nictaba* in 3-week-old *A. thaliana* plants of selected SALK lines designated as KO4 (SALK_007866) and KO6 (SALK_085735C) in comparion to WT plants. Expression analysis was determined by qRT-PCR on a pooled sample of 10 plants; *n*=1; error bars ± SE.

The *F-box-Nictaba* gene *At2g02360* is highly up-regulated in the generated OE lines

Five homozygous transgenic *A. thaliana* lines (T4 generation) for overexpression of F-box-Nictaba protein were selected on kanamycin. qRT-PCR analysis was performed to quantify *F-box-Nictaba* gene overexpression. Fig. 3.8A demonstrates that all five lines overexpress the *F-box-Nictaba* gene. The highest expression is presented by lines OE9 and OE6, reaching almost 300- and 250-fold up-regulation, respectively. Lines OE2, OE4 and OE11 show lower (but still very high) overexpression of the *F-box-Nictaba* reaching transcript levels of approximately 80-100 times higher than in the WT plants.

Two lines, OE4 and OE6, showing different levels of *F-box-Nictaba* gene overexpression were selected for further phenotypic analyses of transgenic plants. Prior to experiments, both lines were checked by Western blot for the presence of elevated amounts of the F-box-Nictaba protein. As shown in Fig. 3.8B, a distinct band of approximately 70 kDa was visible in protein extracts of the OE6 line. No clear and distinct signal was detectable in the protein extract of OE4 plants. Nonetheless, as revealed by qRT-PCR analysis (Fig. 3.8A), the overexpression level of *F-box-Nictaba* in line OE4 is approximately 2.5 times lower than that in the OE6 line. Therefore, the Western blot analysis has been repeated with double amount of total protein loaded. Although the background signal increased due to the high protein load, a separate band of 70 kDa was distinguishable in the OE4 lane

but not in the protein extracts from both the KO6 and WT plants (Fig. 3.8C). Therefore, despite relatively low levels of recombinant protein synthesis, both OE lines overexpress F-box-Nictaba and were considered suitable for further experiments. Interestingly, the molecular weight (MW) of the expressed protein is over 2 times higher than the calculated size for F-box-Nictaba (31.3 kDa).

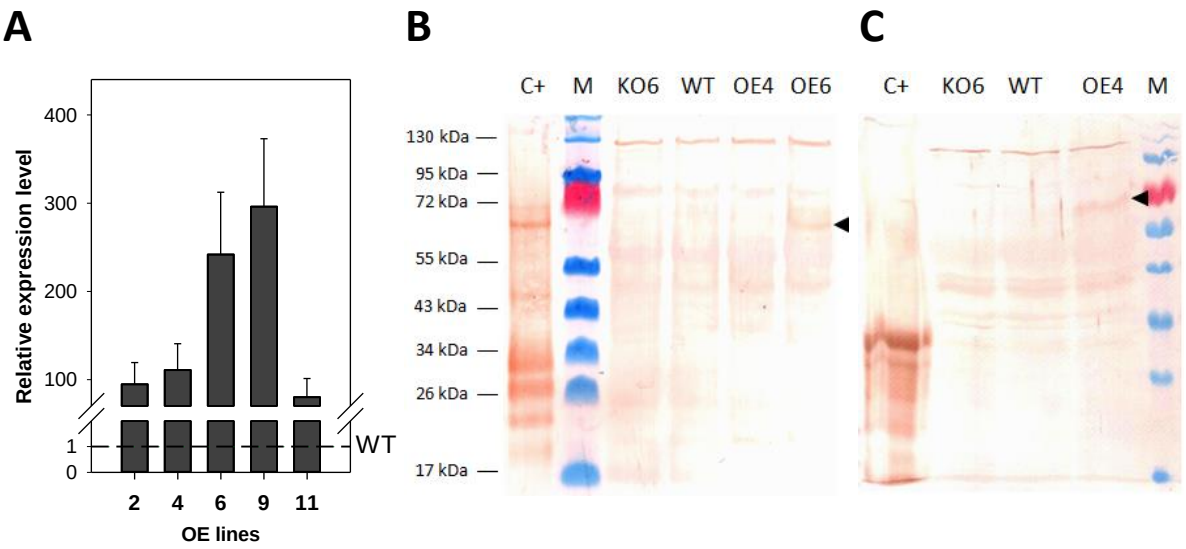


Fig. 3.8 Expression analysis of *F-box-Nictaba* gene and protein in 3-week-old *A. thaliana* OE plants. A, Relative expression of *F-box-Nictaba* in five different OE lines in comparion to WT plants. Expression analysis was determined by qRT-PCR on a pooled sample of 10 plants; *n*=1; error bars ± SE. B-C, Western blot of total protein extracts from WT *A. thaliana* plants and transgenic knockout (KO6) and overexpression (OE4 and OE6) plants immunodetected with the anti-F-box-Nictaba antibody. B, Immunodetection of F-box-Nictaba in 50 µg of total protein extracts from WT, KO6 as well as OE4 and OE6 plants. C, Immunodetection of F-box-Nictaba in 100 µg of total protein extracts from WT, KO6 and OE4 plants. M: protein marker; C+: positive control - purified Nictaba domain of F-box-Nictaba (1 µg). The position of the polypeptide presumably corresponding to F-box-Nictaba is indicated with black marker.

3.4.7.3 Transgenic plants perform similar as WT plants during normal growth conditions

Neither the KO6 line, nor the two overexpression lines showed an obvious altered phenotype throughout development of plants grown under optimal conditions. *In vitro* germination assays did not reveal any differences in transgenic seed germination rates in comparison to the seeds of WT *A. thaliana* plants (Fig. 3.9A-B). Similarly, measurements of the size of fully developed rosette leaves showed that altered *F-box-Nictaba* gene expression did not affect vegetative development of Arabidopsis plants (Fig. 3.9C-D).

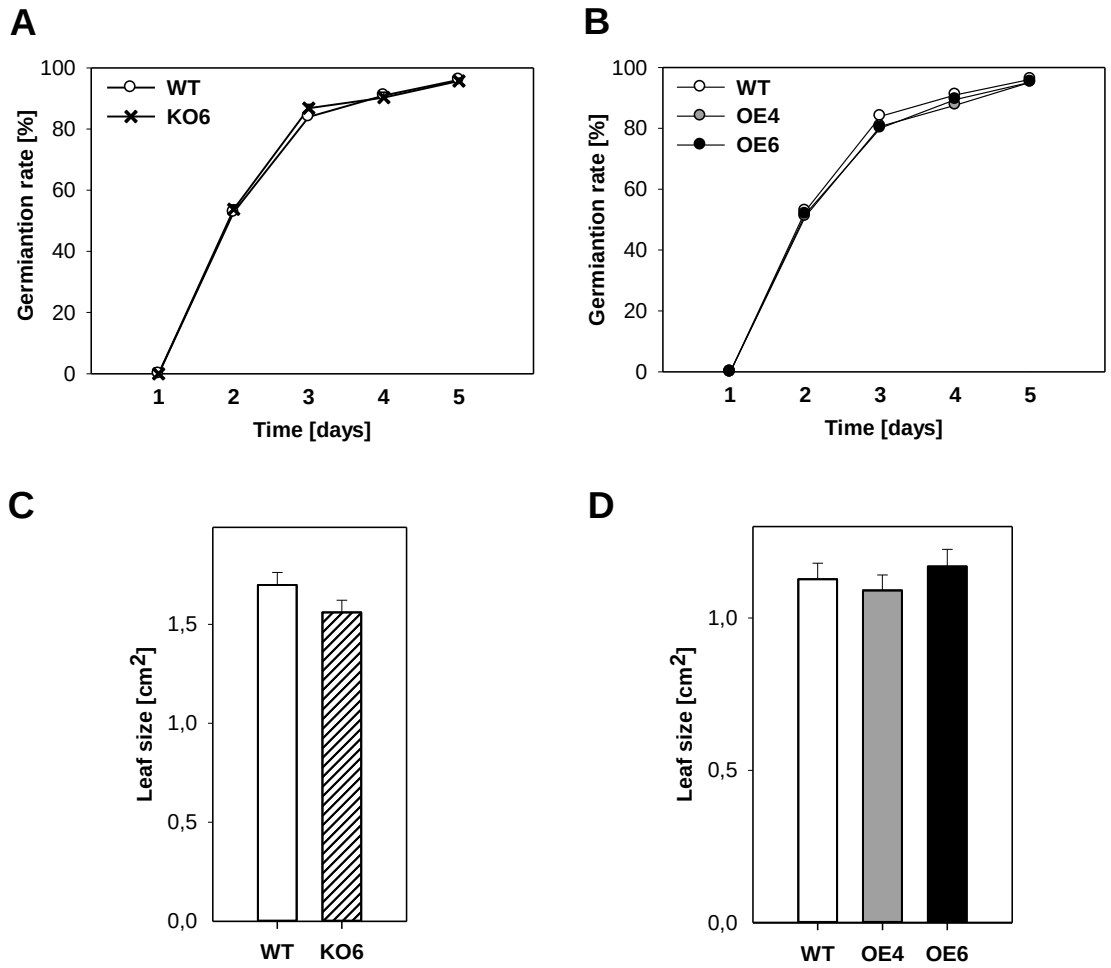


Fig. 3.9 Phenotypic analyses of transgenic plants with altered *F-box-Nictaba* expression. A and B, Assessment of seed germination rates performed by daily calculation of the percentage of germinated seeds starting from the first day of incubation in the growth chamber until day 5. The analysis included two independent biological replicates. *n*=2; error bars ± SE. C and D, Measurement of rosette leaf size in 5-week-old plants. The analysis included two independent biological replicates. *n*=2; error bars ± SE. Statistical analyses were performed using Student's t-test ($p<0.05$).

3.4.7.4 Transgenic *A. thaliana* plants with altered F-box-Nictaba expression show differential *F-box-Nictaba* and *Hsp70b* gene expression after heat stress

Based on the qRT-PCR gene expression analysis, which revealed *F-box-Nictaba* up-regulation after treatment with heat stress (Fig. 3.1C), thermotolerance experiments were performed on transgenic *A. thaliana* plants with altered *F-box-Nictaba* expression. Basal thermotolerance assays included plant treatment with heat stress at 45˚C for 1 h with subsequent recovery at 21˚C for 6 days. For acquired thermotolerance experiments, plants were initially pretreated with 37˚C for 1 h and were allowed to recover for 3 h at 21˚C. Then, the plants were exposed to 45˚C for 1 h with subsequent recovery at 21˚C for 6 days.

As depicted in Fig. 3.10, there were no significant differences in plant survival 6 days post heat stress applications between transgenic lines and WT plants, regardless of the type of thermotolerance (basal or acquired) tested. Survival rates after basal thermotolerance experiment were in the range of 10-20%. Hereby, the transgenic line OE6 presented a higher survival rate (approx. 24%) than WT plants (approx. 16%), whereas only 8% of OE4 plants survived the stress treatment (Fig. 3.10B). In contrast, over 90% of plants of each genetic background subjected to the acquired thermotolerance experiment survived the heat stress treatments with neglegible differences between lines tested.

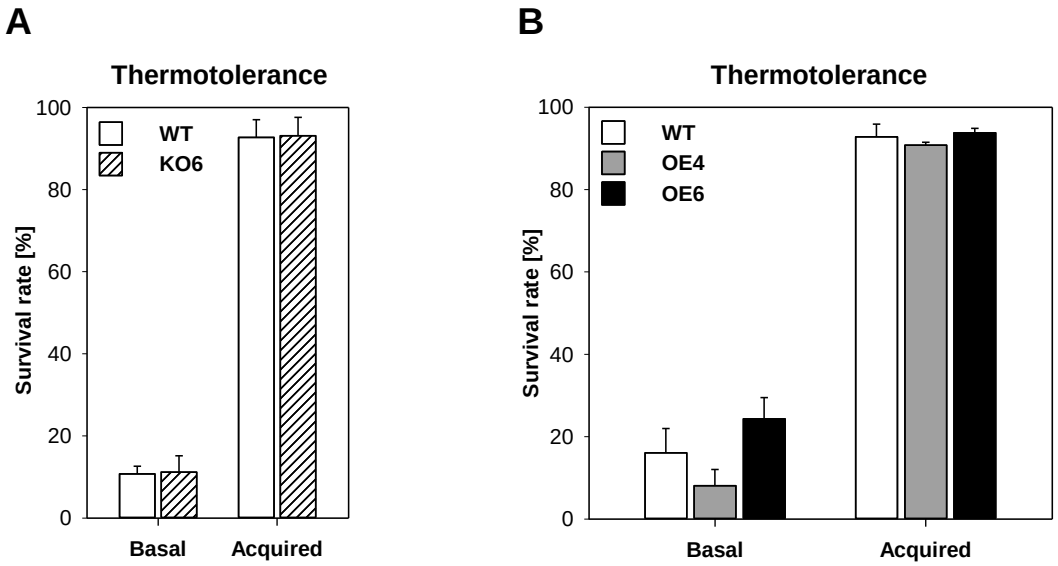


Fig. 3.10 Survival of WT and transgenic *A. thaliana* plants with altered *F-box-Nictaba* expression subjected to basal and acquired thermotolerance experiments. Analysis was performed in two independent biological replicates. *n*=2; error bars ± SE; p<0.05. A, Survival rates of WT and KO6 *A. thaliana* plants determined 6 days after heat stress treatments. B, Survival rates of WT, OE4 and OE6 *A. thaliana* plants plants determined 6 days after heat stress treatments.

Even though survival rates of transgenic and WT plants treated with heat stress were comparable, some differential gene expression was observed for the *F-box-Nictaba* gene (*At2g02360*) and the *Hsp70b* gene (*At1g16030*). According to Fig. 3.11A, positive control gene *Hsp70b* encoding a heat shock protein 70b was significantly 2-fold down-regulated in KO6 mutant line in comparison to the WT plants after the basal thermotolerance experiment, both after 1h of heat stress as well as 24h after the treatment. No statistically significant differences were shown at the beginning (0h) of the experiment. However, the lower expression level of *Hsp70b* in KO6 in comparison to WT line at 1h and 24h is likewise observed in mock-treated plants, although there the gene is even 10-fold down-regulated in KO6 plants after 24h. Thus the differential *Hsp70b* expression in KO6 plants might be associated with *F-box-Nictaba* KO expression.

F-box-Nictaba transcript levels (Fig. 3.11B) in mock-treated KO6 plants were stable over the timecourse of the basal thermotolerance experiment and remained at a very low level of 0.03-0.04 of

relative expression versus WT plants. Interestingly, after heat stress *F-box-Nictaba* expression in the KO6 plants significantly raised more than 10-fold up to the relative expression level of 0.5 at 24h.

Similar to the KO line, despite no effect of *F-box-Nictaba* gene overexpression on the survival of transgenic plants after heat stress, the OE plants demonstrated a slightly altered *Hsp70b* gene expression during the basal thermotolerance assessment (Fig. 3.11C). Untreated OE plants did not show very different expression of the *Hsp70b* gene except for some slight 1.5- to 2-fold higher levels in comparison to WT plants at several of the time points tested. Yet, the *Hsp70b* gene was 9-fold and 3-fold more upregulated in the OE4 and OE6 plants, respectively, in comparison to WT plants 24h after heat stress.

Interestingly, significant alteration of *F-box-Nictaba* expression was observed in the OE plants subjected to heat stress during the basal thermotolerance experiments (Fig. 3.11D). Overexpression levels in both lines gradually significantly decreased as the experiment progressed. This effect is not visible in mock-treated plants for the basal thermotolerance experiment. In OE4 line 20-fold overexpression at the beginning of the experiment decreased to 7-fold up-regulation at 1h in comparison to heat-treated WT plants, while after 24h *F-box-Nictaba* gene expression level in OE4 plants was comparable to the one in the corresponding heat-treated WT plants (Fig. 3.11D). *F-box-Nictaba* transcript levels in line OE6 dropped over 10 times 24h after heat stress in comparison to the mock-treated plants. Since at the same time *F-box-Nictaba* in the WT plants was considerably induced by heat stress, the 600-fold *F-box-Nictaba* overexpression in mock-treated OE6 plants compared to the corresponding WT plants was reduced down to the 4-fold up-regulation level in comparison to WT plants 24 h after heat stress.

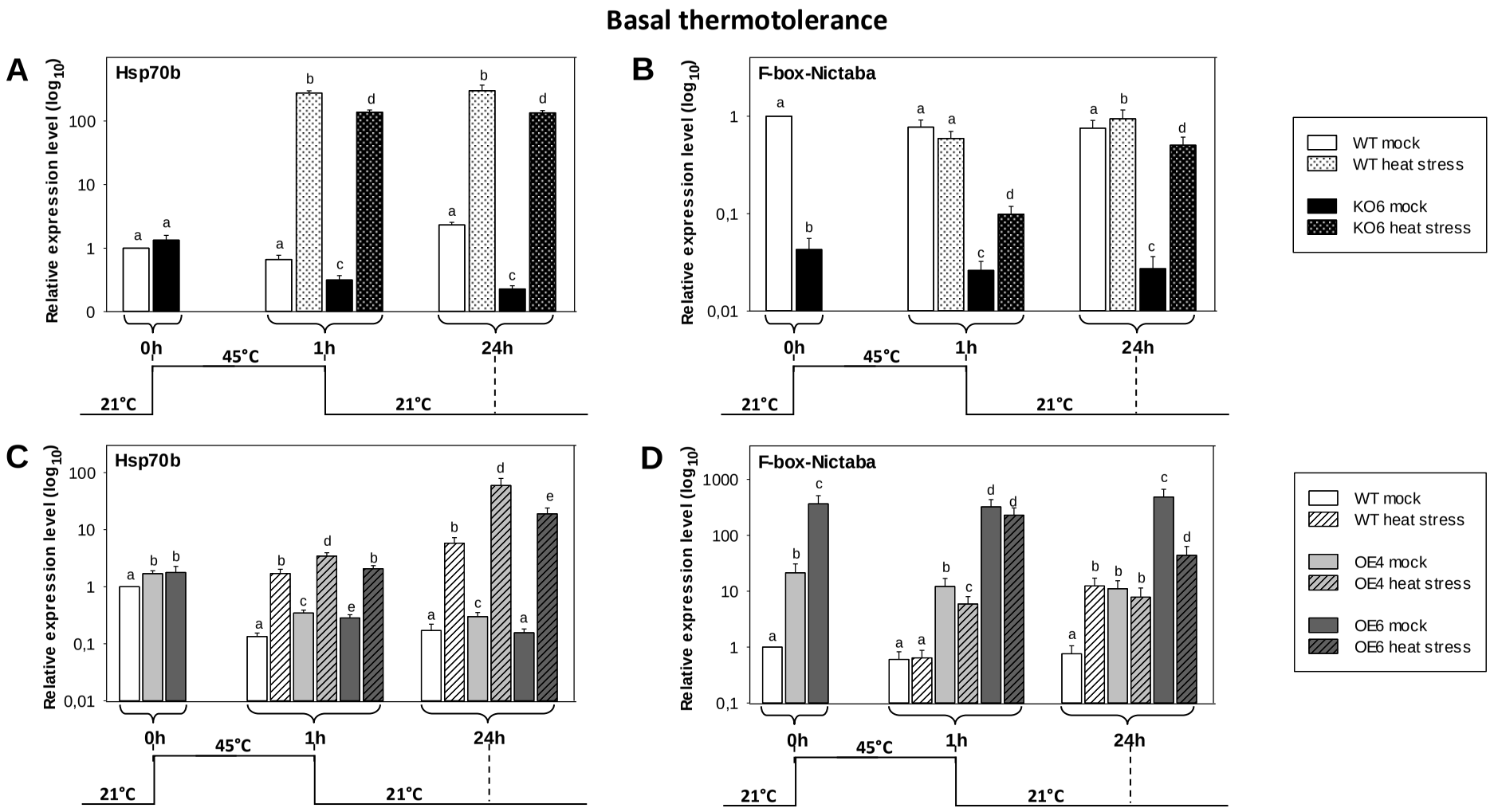


Fig. 3.11 Relative transcript levels of positive control gene *At1g16030* (encoding Hsp70b) and *At2g02360* (encoding F-box-Nictaba) in 7-day-old seedlings of transgenic *A. thaliana* plants during the basal thermotolerance experiment. A and C, Relative expression of *Hsp70b* in KO6 line (A) and in lines OE4 and OE6 (C). B and D, Relative expression of *F-box-Nictaba* in KO6 line (B) and in lines OE4 and OE6 (D). Gene expression levels were calculated relative to the expression in mock-treated WT plants at 0 dpi. For each time point different letters indicate statistically significant differential expression between different lines and compared to mock-treated plants ($p<0.05$). Values were obtained from two independent biological replicates. $n=2$; error bars ± SE.

According to Fig. 3.12A, the positive control gene *Hsp70b* did not show any differential expression in the KO6 line in comparison to WT plants at the beginning of the acquired thermotolerance assessment experiment. However, at 5 h and 24 h, the *Hsp70b* expression was lower in the KO6 line in comparison to WT plants. In turn, at 5 h, the *Hsp70b* expression level was significantly a 4-fold higher in mock-treated KO6 plants compared to mock-treated WT plants.
F-box-Nictaba transcript levels in KO6 plants at the beginning of the acquired thermotolerance experiment were approximately 30 times lower than in the corresponding WT plants, but raised after 1 h at 37°C, and before the final 45°C heat treatment (at 4 h) reached transcript levels comparable to those in WT plants (Fig. 3.12B). After the end of the heat stress treatments *F-box-Nictaba* expression levels dropped in KO6 line down to 0.2 relatively to the expression in WT plants. In general, *F-box-Nictaba* expression levels in mock-treated KO6 plants remained significantly lower than in the mock-treated WT plants during the time course of the acquired thermotolerance experiment, except at 1 h, when the *F-box-Nictaba* expression level in KO6 plants was not significantly different from its level in the WT plants.

OE4 and OE6 lines assessed for acquired thermotolerance presented significantly 2- to 4-fold reduced *Hsp70b* expression levels in comparison to WT plants during the timecourse of the experiment (Fig. 3.12C).
Similar to the basal thermotolerance assessment, *F-box-Nictaba* gene expression changed as the acquired thermotolerance experiment progressed, but the effect was not that tremendous (Fig. 3.12D). In OE4 line the *F-box-Nictaba* expression levels were very variable: 20-fold overexpression dropped to 3-fold up-regulation in comparison to the stress-treated WT plants after pre-treatement with 37°C for 1 h, then the transcript levels increased up to 17-fold overexpression prior to heat shock with 45°C, and finally decreased again down to 3- and 4- fold up-regulation in comparison to WT plants after 1 h of heat shock with 45°C and after 24 h, respectively (Fig. 3.12D). Somewhat similar changes were observed in mock-treated plants of line OE4. In OE6 line 300-350-fold overexpression at 0h was progressively reduced to 45-fold up-regulation level as measured after 24 h (Fig. 3.12D).

In general, the KO line shows lower expression levels of *Hsp70b* in comparison to the WT plants upon heat stress treatment in both thermotolerance experiments (Fig. 3.11A and 3.12A). In contrast, heat-treated OE lines show higher transcript levels of this gene compared to heat-treated WT plants during the basal thermotolerance assay (Fig. 3.11C). However, in the acquired thermotolerance experiment, *Hsp70b* expression levels in the heat-stressed OE lines were usually significantly lower than in the corresponding heat-treated WT plants (Fig. 3.12C).

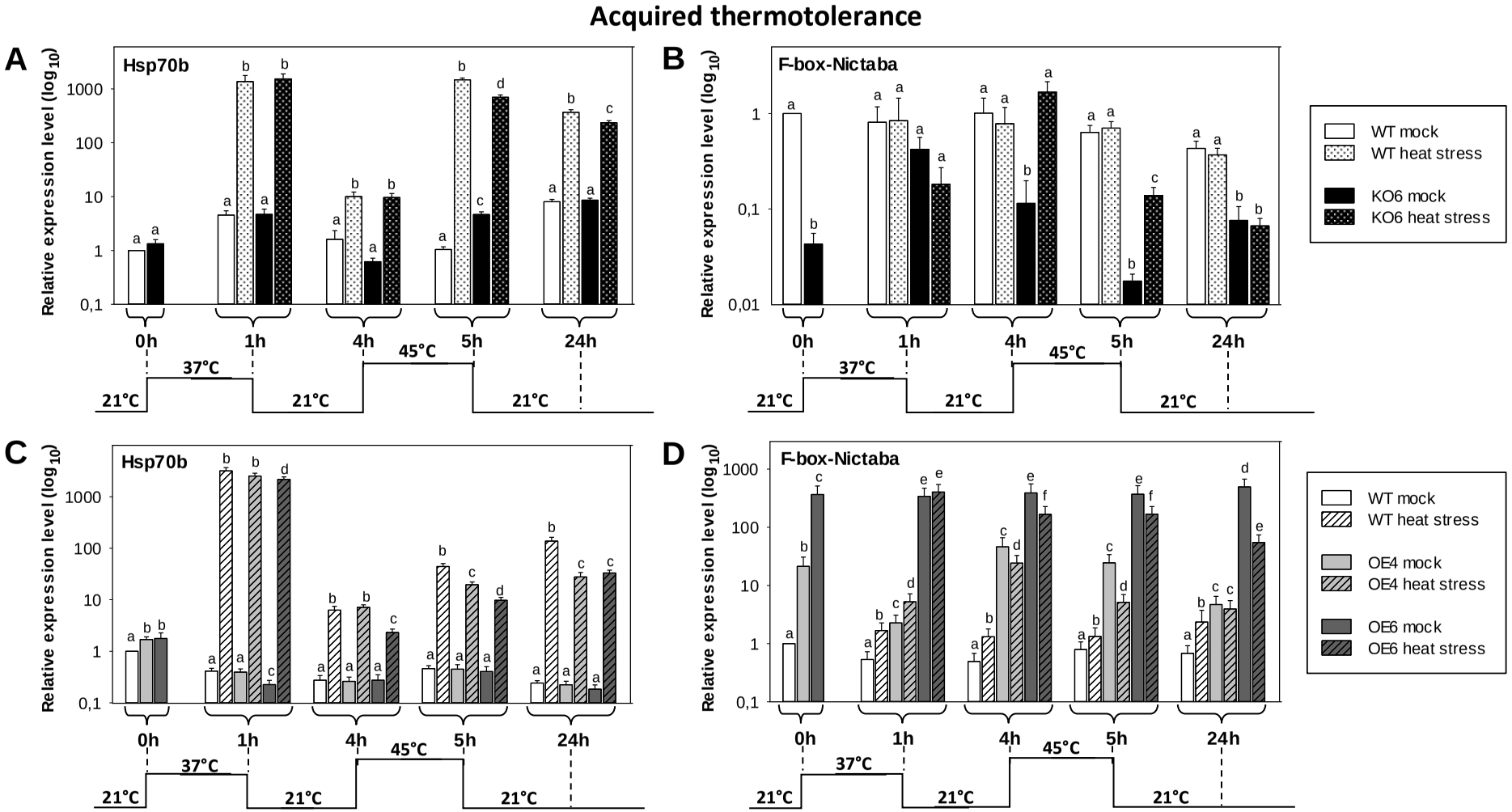


Fig. 3.12 Relative transcript levels of positive control gene *At1g16030* (encoding Hsp70b) and *At2g02360* (encoding F-box-Nictaba) in 7-day-old seedlings of transgenic *A. thaliana* plants during the acquired thermotolerance experiment. A and C, Relative expression of *Hsp70b* in KO6 line (A) and in lines OE4 and OE6 (C). B and D, Relative expression of *F-box-Nictaba* in KO6 line (B) and in lines OE4 and OE6 (D). Gene expression levels were calculated relative to the expression in mock-treated WT plants at 0 dpi. For each time point different letters indicate statistically significant differential expression between different lines and compared to mock-treated plants ($p<0.05$). Values were obtained from two independent biological replicates. $n=2$; error bars ± SE.

3.4.7.5 Plants overexpressing F-box-Nictaba show less damage after *P. syringae* infection than WT plants

As mentioned above, qRT-PCR revealed *F-box-Nictaba* up-regulation after treatment with SA as well as after bacterial infection (Fig. 3.1B and 3.2C). To investigate the role of F-box-Nictaba in plant defense responses against pathogens, WT plants and transgenic *A. thaliana* plants with altered *F-box-Nictaba* expression levels were infected with the virulent hemibiotrophic bacterium *Pst* DC3000. Since infection results in gradual appearance of chlorotic lesions on the rosette leaves, plant damage was assessed by measuring a percent ratio of lesion area relative to the total leaf area. Rosette leaves were collected prior to infection (0 dpi) as well as 3 dpi (when the first symptoms of infection become visible) and 4 dpi.

As demonstrated in Fig. 3.13A-B, the infection experiment on KO6 line versus WT plants was successful and lead to clear disease symptoms characterized by leaf lesions constituting up to 20 % and 30 % of total leaf area at 3 and 4 dpi, respectively. Nevertheless, no significant differences in lesion size could be observed between the KO6 line impaired in *F-box-Nictaba* expression and WT plants.

Although the infection experiment on the OE lines was successful, it resulted in weaker disease symptoms in both transgenic and WT plants when compared to the previous setup (Fig. 3.13C-D). At 3 dpi, lesions occupied 4 and 5 % of total leaf area in OE4 and OE6 plants and 6 % in WT plants, but no significant differences were detected (Fig. 3.13D). In contrast, at 4 dpi, lesions became clearly visible in WT plants and were particularly localized next to the main leaf vein at the site of connection between petiole and lamina, while symptoms where much less obvious for OE plants (Fig. 3.13C). Based on lesion area measurements (Fig. 3.13D), OE4 and OE6 plants developed significantly reduced lesions 4 dpi (at the level of 6.9 and 5.6 %) in comparison to the WT plants.

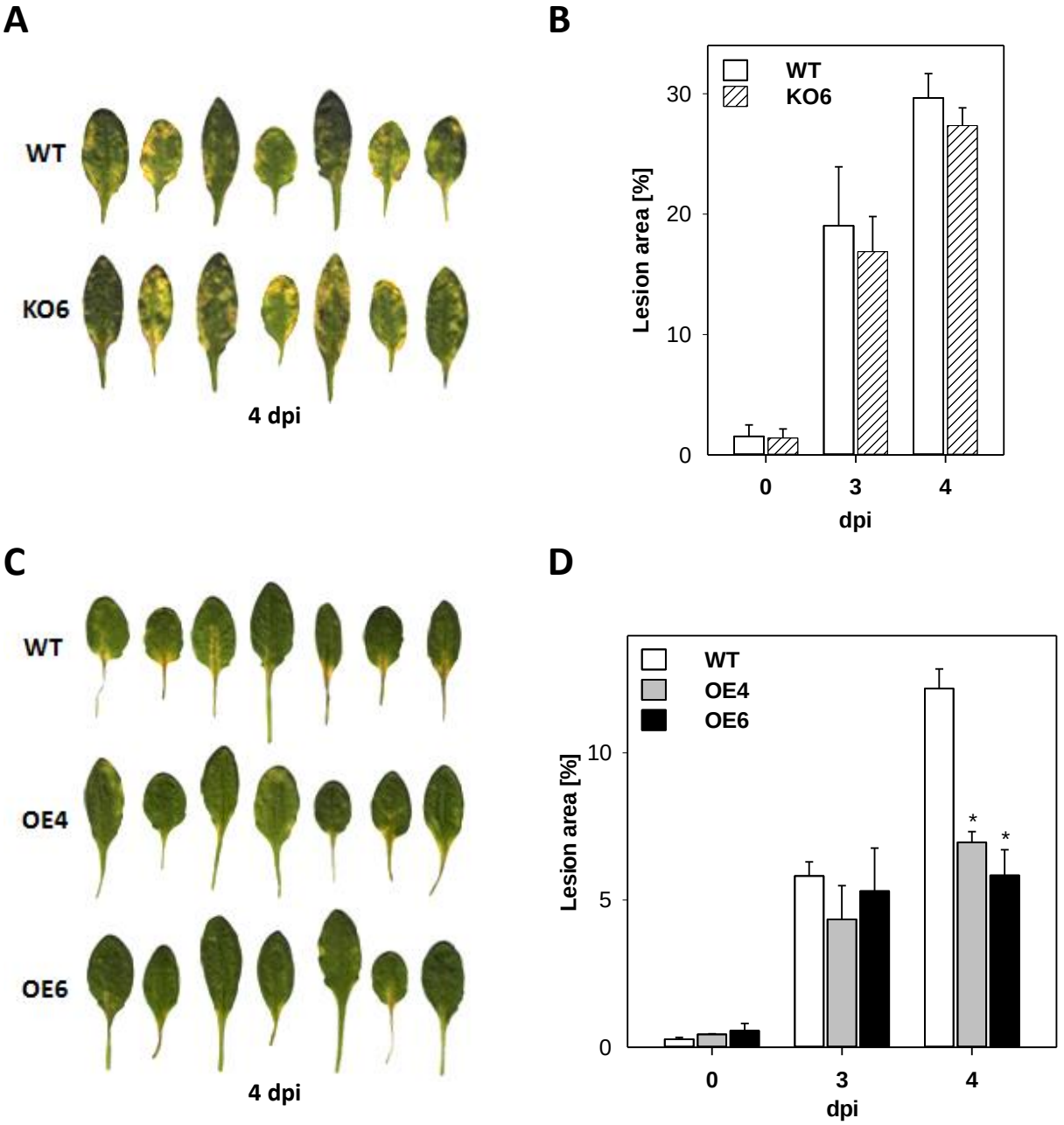


Fig. 3.13 Development of disease symptoms in WT and transgenic *A. thaliana* plants after infection with *Pst* DC3000. A and C, Selected images of leaves collected from infected plants and analyzed 4 dpi. B and D, Disease symptoms in infected WT, KO6, OE4 and OE6 *A. thaliana* plants measured at indicated time points as percent ratio of chlorotic lesion area relative to the total leaf area. Analysis was performed for two independent biological replicates. *n*=2; error bars ± SE. Asterisks indicate statistically significant differences compared to the WT plants ($p<0.05$).

qRT-PCR analysis demonstrated no differential expression for any of the two positive controls (*WRKY70* and *PR1*) neither in infected nor in the mock-treated KO6 plants (Fig. 3.14A-B). Also *F-box-Nictaba* expression remained stable and very low (20-100-fold lower than in the WT plants) throughout the whole experiment (Fig. 3.14C).

On the other hand some differences were observed in the OE plants. *WRKY70* gene was significantly 2-fold down-regulated in both OE lines 0 dpi as well as 1 dpi in infected plants (Fig. 3.14D). At 2 dpi, however, *WRKY70* expression in both lines increased and was 4 and 3 times higher in OE4 and OE6 lines, respectively, in comparison to the infected WT plants. However, some raise in *WRKY70* transcript levels was also revealed in the mock-treated OE6 plants. As presented in Fig. 3.14E, *PR1* levels in the mock-treated OE plants were generally significantly lower down to 7 times in comparison to the WT plants. But, following *Pst* DC3000 infection its transcript levels become comparable to those in WT plants (except for some down-regulation in OE4 line at 2 dpi and in OE6 line at 4 dpi). *F-box-Nictaba* expression levels in OE4 line remained relatively stable over the timecourse of the infection experiment with 150-200-fold overexpression versus corresponding WT plants (Fig. 3.14F). Finally, even though it seems that overexpression of *F-box-Nictaba* in line OE6 progressively decreases over time, a similar trend is observable in the mock-treated plants, and as such the effect is not associated with *Pst* DC3000 infection.

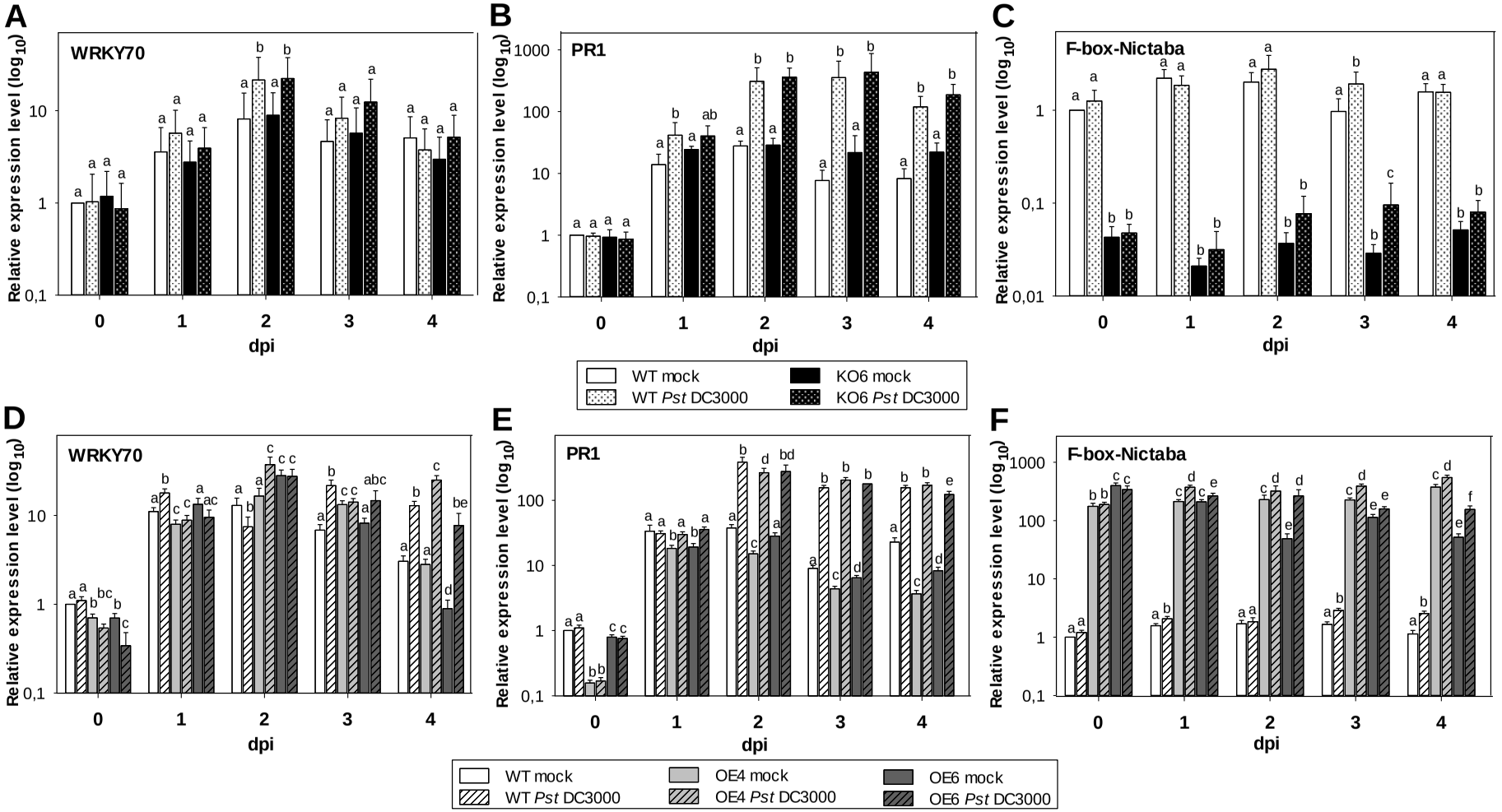


Fig. 3.14 Relative ranscript levels of *At2g02360* (encoding F-box-Nictaba) and selected positive control genes determined by qRT-PCR in 5-week-old transgenic *A. thaliana* plants after *Pst* DC3000 infection. A and D, Relative expression of *At3g56400* (encoding WRKY70) in KO6 line (A) and in lines OE4 and OE6 (D). B and E, Relative expression of *At2g14610* (encoding PR1) in KO6 line (B), and in lines OE4 and OE6 (E). C and F, Relative expression of *At2g02360* (encoding F-box-Nictaba) in KO6 line (C), and in lines OE4 and OE6 (F). Gene expression levels were calculated relative to the expression in mock-treated WT plants at 0 dpi. For each time point different letters indicate statistically significant differential expression between different lines and compared to mock-treated plants ($p<0.05$). Values were obtained from two independent biological replicates. $n=2$; error bars ± SE.

3.5 Discussion

In the past years our knowledge on UPS-mediated protein degradation in plants has widely expanded and it appears to be a critical regulatory mechanism for most cellular and physiological processes in plants (Vierstra 2009; Dielen *et al.*, 2010). In this report we show an extended expression profiling of the glycan-binding F-box-Nictaba protein from *A. thaliana*, integrating qRT-PCR, GUS histochemical assays and gene (co)-expression meta-analyses as well as phenotypic analyses of transgenic plants overexpressing F-box-Nictaba or impaired in its synthesis. The data presented strongly suggest the involvement of F-box-Nictaba in plant defense responses.

3.5.1 *F-box-Nictaba* is a stress-inducible gene responsive to SA, bacterial infection and heat stress

During normal growth and development of WT *A. thaliana* plants, the expression of the *F-box-Nictaba* gene was not particularly pronounced at neither of the developmental stages nor in any tested tissue sample (Fig. 3.1A). This result is in good agreement with the meta-analysis database search performed through Genevestigator (supplementary Fig. A3.4), which revealed a uniform stable *At2g02360* gene expression across the lifecycle of *A. thaliana*. This search also indicated that *F-box-Nictaba* is generally expressed at low to medium levels, indicating that a basal expression of *At2g02360* is required throughout plant development.

Nevertheless, an extensive expression profiling of the *F-box-Nictaba* gene upon treatments with different plant hormones and abiotic stresses revealed that its stable expression is noticeably changed after treatment with SA as well as after heat stress (Fig. 3.1B-C), indicating that the protein might be involved in stress signaling pathways.

Treatment with SA, a plant hormone critical for defense responses against pathogens (Vlot *et al.,* 2009), resulted in an up-regulation of *F-box-Nictaba* gene expression with a gradual increase in transcript levels reaching the maximal 4-fold rise after 10 h, followed by a slight drop in transcript levels after 24 h of SA application (Fig. 3.1B). The fold change in expression was slightly higher than shown by the microarray data available on the eFP browser (Winter *et al.*, 2007). As indicated in the Table A3.4, the *At2g02360* promoter sequence contains multiple W-box-containing regions, corresponding to *cis*-regulatory elements [(C/T)TGAC(C⁄T)] specifically recognized and bound by the plant-specific family of WRKY transcription factors (Yamasaki *et al.,* 2012). Since most WRKY proteins are involved in plant stress responses (Rushton *et al.*, 2010) including pathogen infection (Eulgem and Somssich, 2007; Pandey and Somssich, 2009), it is highly probable that inducible expression of *F-box-Nictaba* upon SA treatment is under the control of (a) SA-dependent WRKY transcription

regulator(s). This is also supported by the expression profile of the positive control gene *WRKY70*, which encodes a SA-inducible protein activating downstream expression of defense-related genes encoding e.g. PR proteins (Li *et al.*, 2004; Ren *et al.*, 2008b). *WRKY70* was up-regulated much faster than *F-box-Nictaba* and reached a peak expression already after 3 h of SA application (Fig. 3.1B). This delay in maximal expression for the *F-box-Nictaba* gene in comparison to one of the key SA-dependent WRKY transcription factors suggests that *F-box-Nictaba* is a late SA-responsive gene. The search for the genes co-expressed with *F-box-Nictaba* (Fig. 3.4 and Table 3.1) revealed a *WRKY25* gene (*At2g30250*). WRKY25 is a stress- and SA-inducible transcription factor shown to recognize W-box sequences and to function as a negative regulator of SA-mediated defense responses to *P. syringae* (Zheng *et al.,* 2007). Expression analysis of the *F-box-Nictaba* gene in *WRKY*-specific mutant lines could bring more definite insights in the mode of transcriptional regulation of *F-box-Nictaba* and could show if expression relies on the upstream induction of WRKY transcription regulator(s).

Next, as depicted in Fig. 3.2C, infection of WT *A. thaliana* plants with the virulent *Pst* DC3000, a hemibiotrophic bacterium pathogenic to *A. thaliana* and activating the SA-dependent plant defense pathway (Katagiri *et al.*, 2002; Glazebrook, 2005; Nomura *et al.*, 2005), resulted in a 2.6-fold increase in *F-box-Nictaba* gene expression. This transcript level is only slightly lower than the one observed after treatment with SA (Fig. 3.1B) and comparable to the microarray expression data available on the eFP browser (Winter *et al.*, 2007). As expected, *Pst* DC3000 strongly enhanced the expression of two control genes, encoding the SA-inducible transcription factor *WRKY70* (Fig. 3.2A) and *PR1* (Fig. 3.2B) a well documented WRKY70-regulated marker of SA-dependent responses and SAR (Li *et al.*, 2004; Ren *et al.*, 2008b; Vlot *et al.*, 2009). In contrast, infection with the necrotrophic fungus *B. cinerea* strain B05.10, which activates a JA/ET-dependent plant resistance pathway (Thomma *et al.*,1999; Wasternack and Hause, 2013), only slightly affected *F-box-Nictaba* mRNA levels by down-regulation of *At2g02360* 2 and 3 dpi (Fig. 3.2F). Consistently, MeJA and ET, the two hormones involved in the plant signaling pathway upon fungal infection, initially also had a minor down-regulating effect on *F-box-Nictaba* expression (supplementary Fig. A3.2). The mutual antagonism in stress signaling cross-talk between SA and JA is well documented (Pieterse *et al.*, 2012; Thaler *et al.*, 2012). Whereas *B. cinerea* generally triggers the JA/ET-dependent pathway (Thomma *et al.,* 1999), El Oirdi *et al.* (2011) demonstrated in tomato that the fungus can activate at the same time SA-dependent defenses, which suppresses the JA-induced defenses by hormone antagonism, ultimately leading to increased plant susceptibility. However, in our experiments the JA/ET-responsive positive control gene *PDF1.2* (Thomma *et al.,* 1999) was highly up-regulated (Fig. 3.2D), confirming the activation of the JA/ET-signaling pathway. It has previously been shown that *B. cinerea* also significantly induces *PR1* expression in *A. thaliana* (Ferrari *et al.,* 2003; La Camera *et al.,* 2011). However, the influence of *B. cinerea* infection on the levels of *PR1* expression can vary greatly, since for instance Cabot *et al.* (2013) reported only 3-fold increase in *PR1* expression during such experiment in Arabidopsis. In this work the expression of the SA pathway marker *PR1* was not significantly affected in *A. thaliana* by *B. cinerea* (Fig. 3.2E), thus there was no noticeable activation of the SA-dependent defenses. Since *F-box-Nictaba* expression was up-regulated in WT *A. thaliana*

plants after SA treatment and *Pst* DC3000 infection but not upon MeJA/ethephon treatment or infection with *Botrytis,* it is reasonable to conclude that F-box-Nictaba is playing a role in SA-mediated plant defense reactions to pathogen infection. Accordingly, the co-expression data as presented in Fig. 3.4 and Table 3.1 show *F-box-Nictaba* co-expression in a network of defense-related genes. Interestingly, two of them, *At1g64280* (encoding regulatory protein NPR1) and *At4g19660* (encoding regulatory protein NPR4), are both key regulators of plant responses against bacterial pathogens and their function relies on or is modulated by the UPS (Fu *et al.,* 2012; Spoel *et al.*, 2009). Notably, the NPR1 protein is the direct activator of several WRKY transcription factors (Spoel *et al.*, 2009; Wang *et al.*, 2006). In turn, NPR4 has been demonstrated to be one of the SA receptors which functions as an adaptor of the Cullin3 Ub ligase to mediate NPR1 degradation in a SA-regulated manner (Fu *et al.,* 2012). Also, two strongly co-expressed genes *At3g28940* and *At3g28950* (revealed by all four screened databases), are encoding the AIG2 (avrRpt2-induced gene 2)-like proteins possibly involved in plant responses to pathogen infection (Reuber and Ausubel, 1996).

As delineated from Fig. 3.1C, *F-box-Nictaba* was up-regulated not only after pathogen infection but also after heat stress with a similar, up to 3-fold increase in expression. Here, the positive control gene *Hsp70b*, encoding a heat shock-responsive chaperone preventing protein aggregation and mediating protein folding (Sung *et al.*, 2001), demonstrated a fast and high up-regulation. As such, it appears that F-box-Nictaba expression is responsive towards both biotic as well as abiotic stresses, which is in good agreement with the gene expression data from the ATH1 affymetrix microarray available at the eFP browser (Winter *et al.*, 2007). This finding should not be surprising since the cross-talk between biotic and abiotic stress signaling pathways is quite common and has been extensively studied in recent years (Cao *et al.*, 2011; Lee and Luan, 2012; Cheng *et al.*, 2013; Xiao *et al.*, 2013). There is also considerable evidence emerging for the relation between pathogen infection and thermotolerance in plants (Clarke *et al.*, 2004; Cronjé *et al.*, 2004; Snyman and Cronjé, 2008). The points of convergence between different pathways are usually transcription factors (e.g. WRKYs) and kinases which are molecular players common to multiple networks and integrating different stress signals to fine-tune plant responses towards environmental stress. *F-box-Nictaba* up-regulation upon heat stress might thus be driven by heat shock-activated transcription factors which could bind to the heat shock-responsive *cis*-elements (CCAATBOX1) present in the *At2g02360* promoter sequence (supplementary Table A3.4), but also by heat stress-induced WRKY transcription factors (Rushton *et al.*, 2010) activating *F-box-Nictaba* expression through the multiple W-box motifs [(C/T)TGAC(C/T)] in its promoter sequence.

3.5.2 *F-box-Nictaba* expression is pronounced in non-glandular *A. thaliana* trichomes

As presented in Fig. 3.3B, GUS histochemical assays showed preferential activity of the *At2g02360* promoter in non-glandular leaf trichomes of transgenic *A. thaliana* plants. The highly prominent GUS staining in the trichomes present mostly on young leaves was visible throughout the development of the plants and was consistent in all independent transgenic lines tested. Non-glandular trichomes are

epidermal hairs on the surface of most plant aerial organs, characterized by a unicellular dendritic structure with a stalk and three to four branches. They are implicated in transpiration control, thermotolerance and protection against insects, diseases and UV irradiation (Johnson, 1975; Mauricio and Rausher, 1997; Traw and Bergelson, 2003; Dalin *et al.*, 2008). Although the non-glandular trichomes are presumably non-secreting, they do express genes involved in biosynthesis of secondary compounds (anthocyanins, flavonoids and glucosinolates) suggesting their putative role in plant defense against pathogens (Walker *et al.*, 1999; Serna and Martin, 2006; Jakoby *et al.*, 2008; Frerigmann *et al.,* 2012). Transcript profiling of Arabidopsis trichomes revealed WRKY transcription factors in the top genes highly expressed in trichome tissue (Jakoby *et al.*, 2008; Dai *et al.*, 2010). In line with the presumed WRKY-dependent regulation of *F-box-Nictaba,* several putative *cis*-regulatory elements reported to be responsible for trichome-specific gene regulation could be identified in the *F-box-Nictaba* promoter sequence (supplementary Fig. A3.3), including eight MYB-like recognition sites (AACCAAAC) (Ni *et al.,* 2008) and five T/G-box elements (AACGTG) (Shangguan *et al.,* 2008). In view of the intriguing pronounced *F-box-Nictaba* promoter activity in Arabidopsis trichomes, further experiments, which are described in Chapter 4, were performed to corroborate *F-box-Nictaba* expression in these structures.

3.5.3 Altered *F-box-Nictaba* gene expression affects plant responses towards stress

Transgenic *A. thaliana* plants, either deficient in F-box-Nictaba protein or overexpressing it, do not present any clearly distinct phenotype throughout their lifecycle under normal growth conditions. Thus, it seems unlikely that F-box-Nictaba is playing an essential role in any of the fundamental processes of plant development and morphogenesis. This is in a good agreement with the relatively stable *F-box-Nictaba* expression in WT *A. thaliana* plants during development and among different tissues (Fig. 3.1A). However, since it has been demonstrated that *F-box-Nictaba* gene expression is induced after treatment with SA, bacterial infection and heat shock (Fig. 3.1B-C and Fig. 3.2C), it was investigated whether transgenic plants would perform differently from WT plants under unfavorable conditions. SA- and pathogen-inducible proteins in plants have been reported to increase plant resistance when recombinantly overexpressed, while their downregulation or knockout resulted in more severe disease development (Dóczi *et al.,* 2007; Shim *et al.,* 2013; Xiao and Chye, 2011). As presented in Fig. 3.13C-D, Arabidopsis plants overexpressing F-box-Nictaba exhibited significantly reduced disease symptoms compared to WT plants 4 dpi with *Pst* DC3000. Thus, it could be anticipated that the KO line should exhibit higher susceptibility to *Pst* DC3000 infection. Yet, no clear differences were seen between the performance of the KO plants and WT plants (Fig. 3.13A-B). As previously reported (Delporte *et al.,* 2015; Lannoo *et al.,* 2008), the *A. thaliana* genome contains more than 20 homologous genes encoding F-box-proteins with a Nictaba domain for which evidence of expression is available. They show relatively high AA sequence similarity with F-box-Nictaba encoded by *At2g02360* in both their F-box domain as well as Nictaba domain at the level of >90% and > 40%, respectively. Therefore, it is likely that some of these genes show functional redundancy

and could fulfill the same or at least a similar role, e.g. by recognizing common targets for degradation. For instance, protein redundancy with respect to the function in plant resistance to *Pst* DC3000 infection has been demonstrated by Maekawa *et al.* (2012) for two Ub ligases from Arabidopsis, i.e. ATL31 and ATL6, sharing 65% of sequence identity in their AA sequences. While overexpression of either of them provided increased resistance to the plants, separate single mutants did not reveal any robust phenotype after infection. However, increased susceptibility to *Pst* DC3000 infection was observed in double mutant plants. Also, no clear phenotype was reported for the Arabidopsis mutants impaired in the synthesis of the SA- and pathogen-inducible legume lectin-like protein SAI-LLP1, while its overexpression limited proliferation of the avirulent strain *Pst* Avr-Rpm1 (Armijo *et al.,* 2013). Since there is evidence for basal expression of genes encoding other SAI-LLP1-like proteins in Arabidopsis, functional redundancy has been suggested as the possible cause underlying the results. Recently, the functional redundancy has been discussed for the Arabidopsis group of F-box protein genes expressed during male gametogenesis (Ikram *et al.,* 2014). Consequently, although the F-box-Nictaba protein encoded by the *At2g02360* is involved in plant defense responses to pathogen infection, it will not play an essential role on its own, but will rather act as one partner in a complex network of proteins.

Proteins demonstrated to increase or decrease plant resistance against pathogen infection including *Pst* DC3000 are most often involved in the modulation of expression of PR genes (Dóczi *et al.,* 2007; Lim *et al.,* 2014; Shim *et al.,* 2013; Xiao and Chye, 2011). Thus, aside from the phenotypic analysis of the infected plants, we also investigated the possibility of dissimilarities between WT and transgenic lines by checking the expression levels of the defense marker genes *WRKY70* and *PR1*, an early and a late responsive gene, respectively (Li *et al.*, 2004; Ren *et al.*, 2008b). Hereby, no differences were detected for the KO plants (Fig. 3.14A-B), which was in agreement with the lack of an apparent distinct phenotype for the KO6 line. In contrast, expression of the two genes in both OE lines seemed to be reduced compared to WT plants prior to infection, but increased after inoculation with the pathogen (Fig. 3.14D-E). The beneficial effect of an initial down-regulation of *WRKY70* and *PR1* on the enhanced resistance to *Pst* DC3000 in plants overexpressing F-box-Nictaba is interesting but rather puzzling, and thus requires more in-depth studies to unravel the mechanism behind it.

Finally, we have also performed basal and acquired thermotolerance experiments on the transgenic Arabidopsis plants in view of *F-box-Nictaba* up-regulation after heat stress. While plants characterized by basal thermotolerance are capable of growing under high temperatures, those with acquired thermotolerance can only survive lethal heat stress after initial pre-treatment with a moderate heat application (Clarke *et al.,* 2004; Larkindale *et al.,* 2005). Contrary to the infection assays with *Pst* DC3000, no clear positive or negative phenotype effects could be detected in both basal and acquired thermotolerance tests as survival rates were comparable for all plants (Fig. 3.10). However, remarkable changes have been demonstrated in *F-box-Nictaba* expression (Fig. 3.11B and D as well as 3.12B and D). Even though the overexpression of the *F-box-Nictaba* gene in transgenic plants was very high (approximately 350-fold overexpression in OE6 line) or moderately high (approximately 20-fold overexpression in OE4 line) at the beginning of the thermotolerance assays,

transcript levels in both lines were progressively decreasing as the experiments advanced in time. The reduction in overexpression of *F-box-Nictaba* was mostly pronounced in the basal thermotolerance experiment (Fig. 3.11D), with a reduction of 10 times for the OE6 line 24 h after heat stress in comparison to mock-treated plants. Consequently, at the end of the experiment (24 h post heat shock), OE6 remained an overexpression line with only 4-fold up-regulation of *F-box-Nictaba* in comparison to the heat-stressed WT plants, and the OE4 line had comparable *F-box-Nictaba* mRNA levels to those in the corresponding heat-treated WT plants. No such drastic effects of heat stress on CaMV 35S promoter-driven expression were reported in plants and thermotolerance assessment experiments were successfully performed using this expression system (Chae *et al.*, 2013; Li *et al.*, 2013; Liu and Charng, 2013; Macková *et al.,* 2013). It has been reported though for tissue cultures of the moss *Physcomitrella patens* that over-expression under the 35S promoter is lower when cultures are kept in the dark (Saidi *et al.*, 2009). Nevertheless, Chae *et al.* (2013) also performed heat stress treatments in dark conditions on Arabidopsis plants over-expressing a gene controlled by the 35S promoter. In this case, the thermotolerance assay was reported to be successful. Furthermore, it has to be noted that *F-box-Nictaba* over-expression in the OE lines did not return to its initial levels during the recovery period (21°C in light conditions) as measured at 24 h, but remained significantly reduced.

Thus, since the overexpression levels were remarkably reduced, it is not surprising that a specific phenotype of the presumed overexpression lines was not observed. In contrast, the overexpression of *F-box-Nictaba* in OE4 and OE6 plants was not negatively affected after *Pst* DC3000 infection (Fig. 3.14F) and the transgenic plants presented an increased resistance towards bacterial infection.

Conversely, in KO6 plants which were assessed for thermotolerance, the *F-box-Nictaba* transcript levels were increased up to a similar level detected in WT plants after heat stress (Fig. 3.11B and 3.12B). Although this increased level of transcripts in the KO line is most probably a result of a read-through transcription past the T-DNA insert (Ulker *et al.,* 2008; Wang, 2008; Zubko *et al.,* 2011), the promoter still seems to be responsive to heat stress. Again, as in the case of the *Pst* DC3000 infection, the fact that there cannot be assigned a distinguishable phenotype for the heat-stressed KO plants might result from the protein redundancy within the family of Nictaba-related F-box proteins. Interestingly, KO6 plants subjected to the basal thermotolerance assay showed 2-fold lower expression levels for *Hsp70b* (Fig. 3.11A), while in OE lines this gene becomes significantly more upregulated than in the WT plants (Fig. 3.11C). No such consistent effects were detected in the acquired thermotolerance experiments (Fig. 3.12C).

These findings strongly suggest that F-box-Nictaba is needed during plant responses to heat stress, at certain optimal levels. Although F-box-Nictaba was highly overexpressed in the transgenic plants under the control of the strong CaMV 35S promoter, F-box-Nictaba expression might be tightly regulated post-transcriptionally in Arabidopsis plants. Indeed, the expression of genes can be highly regulated at both transcriptional and post-transcriptional level (Floris *et al.,* 2009; Ruszka *et al.,* 2012). Post-transcriptional gene regulations occur at the levels of pre-messenger RNA (mRNA) processing (capping, splicing, and polyadenylation), mRNA stability, and mRNA translation. Alternative splicing should not be an issue here in the transgenic plants, since only the coding

sequence of the *F-box-Nictaba* gene was cloned under the control of the 35S promoter. Another option could be the regulation of mRNA stability by RNA silencing, a mechanism involved in gene expression control during plant development, responses to viral infection and in response to abiotic stress (Kruszka *et al.,* 2012; Molnar *et al.,* 2011; Wang *et al.,* 2012). It acts through small non-coding RNAs (sRNAs) and can result in cleavage of the target mRNA or in translation repression. sRNAs can be induced or downregulated under specific stress conditions and thereby either repress negative regulators of stress tolerance or allow the accumulation of positive regulators.

Based on the phenotypic analysis of the transgenic plants under pathogen infection and heat stress conditions, it can be concluded that F-box-Nictaba is clearly involved in plant responses towards stress. Nevertheless, it is intriguing how responses of transgenic plants differ depending on whether abiotic stress or biotic challenges are applied. There is an evident crosstalk between SA and heat response pathways, however, the exact molecular mechanism of SA signaling in thermotolerance remains unclear and requires further research (Clarke *et al.,* 2004, 2009*;* Larkindale *et al.,* 2005; Snyman and Cronjé, 2008; Zhang and Wang, 2011).

Chapter 4

Expression analysis in the trichomes of *Arabidopsis thaliana* plants

Part of the manuscript in preparation:

Stefanowicz K, Lannoo N, Zhao Y, Eggermont L, Van Hove J, Al Atalah B, Rydahl MG, Willats WGT, Van Damme EJM. F-box-Nictaba from *Arabidopsis thaliana* is a plant defense protein involved in pathogen infection and heat stress.

4.1 Abstract

Trichomes in Arabidopsis are large cells present as dendritic protuberances on different plant aerial organs. They have been extensively studied as a model structure to investigate the molecular mechanism underlying cell differentiation in plants. Trichomes in Arabidopsis are generally suggested to be involved in plant defense against different environmental stresses, although little is known regarding their exact roles in plant protection. Previously, it has been shown that the promoter of the *At2g02360* gene encoding the Arabidopsis F-box-Nictaba protein exhibits preferential activity in the trichomes. F-box-Nictaba is a lectin capable of binding galactose oligomers as well as *N*- and *O*-glycans containing *N*-acetyllactosamine (LacNAc) structures, Lewis A, Lewis X, Lewis Y and type-1 B antigen motifs. It is suggested to play a role in plant defense responses against pathogen infection presumably by binding specific glycosylated proteins and targeting them for proteasomal degradation. In order to further corroborate the trichome-specific expression of the *F-box-Nictaba* gene we have performed qRT-PCR analysis on trichomes isolated from *A. thaliana* leaves. Our results confirmed the histochemical staining experiments and demonstrated high *At2g02360* transcript levels in the trichomes. Furthermore, also significant amounts of the F-box-Nictaba protein could be immunodetected in the trichomes. Finally, two genes encoding a specific galactosyl- and fucosyltransferase involved in the synthesis of Lewis A structures in Arabidopsis, were demonstrated to have differential expression in the trichomes. Altogether, these findings provide new insights in the physiological role of F-box-Nictaba in plants.

4.2 Introduction

Trichomes are specialized epidermal cells which can be present as protuberances on different plant aerial organs such as leaves, stems, petioles, petals or the seed coat (Johnson, 1975). They are involved in a broad range of biological processes and are commonly associated with plant defense against diverse environmental stress factors. In general, trichomes can be grouped into two distinct types: (1) the simple non-glandular trichomes and (2) the glandular secreting trichomes (Wagner *et al.,* 2004). Since glandular secreting trichomes can exude phytochemicals adversely affecting herbivores, insects and pathogens, they are mostly studied in view of their role in plant protection (Tissier, 2012a). In contrast, the simple type trichomes in Arabidopsis are considered as an excellent model to investigate the molecular mechanism underlying cell differentiation and pattern formation in plants (Pesch and Hülskamp, 2009; Schellmann and Hülskamp, 2005). Arabidopsis trichomes are large unicellular cells characterized by a typical dendritic structure with a stalk and three to four branches, present and well distinguishable (even by naked eye) on most aerial organs, with exception of hypocotyls and cotyledons.

Trichomes originate from the protodermal cells in primordia of developing leaves. While surrounding epidermal cells continue normal division, the cells destined to develop into trichomes cease to divide and undergo at least four endoreduplication cycles with DNA replication but without mitosis or

cytokinesis (Walker *et al.,* 2000). Consequently, the trichome cell size increases, changes its growth direction into perpendicular to the leaf surface and undergoes branching. As a result, a mature branched trichome has an average DNA content of 32C (Hülskamp *et al.,* 1994; Schnittger and Hülskamp, 2002). The production, morphology, distribution and number of trichomes is spatially and temporally regulated. For instance, their morphology varies between different organs: trichomes on rosette leaves contain three to four branches, whereas those present on cauline leaves and stems are less branched or completely unbranched, respectively (Telfer *et al.,* 1997).

Over the years evidence has accumulated that trichome development in Arabidopsis is based on a highly complex and tightly controlled regulatory network of over 30 different genes, including multiple transcriptional activators and repressors (Pattanaik *et al.,* 2014; Schellmann and Hulskamp, 2005). This network comprises three major groups of transcription factors activating trichome development: R2R3 MYBs (including MYB0/GLABROUS1 (GL1) and MYB82; Kirik *et al.,* 2001, 2005; Liang *et al.,* 2014), a basic helix-loop-helix (bHLH) factor (including paralogs GLABROUS3 (GL3), ENHANCER OF GLABROUS3 (EGL3), TRANSPARENT TESTA 8 (TT8) and AtMYC1; Maes *et al.,* 2008; Payne *et al.,* 2000; Symonds *et al.,* 2011; Zhang *et al.,* 2003; Zhao *et al.,* 2012b), and a WD40 repeat (WDR) protein (TRANSPARENT TESTA GLABRA 1 (TTG1; Walker *et al.,* 1999). Altogether, these regulatory proteins form a trimeric MBW complex MYB(GL1)-bHLH(GL3/EGL3)-WDR(TTG1) which triggers expression of the homeodomain protein called GLABROUS2 (GL2) responsible for inducing trichome formation (Rerie *et al.,* 1994).

Seven single repeat R3 MYBs act as negative regulators of trichome development and include TRIPTYCHON (TRY), CAPRICE (CPC), ENHANCER OF TRY and CPC 1, 2 and 3 (ETC1,2,3), TRICHOMELESS 1 and 2 (TCL1,2) (Gan *et al.,* 2011; Kirik *et al.,* 2004a, 2004b; Schellmann *et al.,* 2002; Schnittger *et al.,* 1999; Wada *et al.,* 1997, Wang *et al.,* 2007; Wester *et al.,* 2009). These negative regulators compete with the R2R3 MYBs for binding the bHLH factor to form a repressor complex (Ishida *et al.,* 2008; Yang and Ye, 2013; Wang and Chen, 2014).

In parallel with the major activator–repressor system, other mechanisms may also modulate trichome development. In a depletion mechanism the bHLH factor traps the intercellularly transported activator protein TTG1, resulting in the lack of TTG1 in neighboring cells and development of bHLH-TTG1-rich cells into trichomes (Bouyer *et al.,* 2008; Balkunde *et al.,* 2011). Moreover, the activatory MBW complex up-regulates the expression of genes encoding the repressors (TRY/CPC). These repressors can move to neighboring cells to form a repressor complex inhibiting trichome formation. Apart from the core MYB-bHLH-WDR TFs, a group of C2H2 zinc finger transcription factors has been implicated in positive regulation of trichome development (Yan *et al.,* 2014).

Phytohormones such as GA_3, JA, cytokinins, BR and SA play an important role in trichome formation in Arabidopsis (Perazza *et al.,* 1998; Traw and Bergelson, 2003; Gan *et al.,* 2007a, 2007b; Maes *et al.,* 2008; Qi *et al.,* 2011, 2014; Yoshida *et al.,* 2009). GA_3, JA and wounding (stress associated with JA-signaling) as well as cytokinins trigger trichome formation in plants through up-regulation of key transcription factor genes (Maes *et al.,* 2008; Perazza *et al.,* 1998; Traw and Bergelson, 2003). In

contrast, SA is a negative regulator of trichome development and significantly reduces the number and density of trichomes in Arabidopsis (Traw and Bergelson, 2003). This negative effect is associated with the well-described antagonistic interaction between JA and SA-dependent pathways in Arabidopsis (Gimenez-Ibanez and Solano, 2013; Pieterse *et al.,* 2012; Thaler *et al.,* 2012).

Even though the research on Arabidopsis trichomes is mainly focused on cell differentiation and pattern formation, trichomes have also been reported to play an important defensive role in plants. Trichomes in Arabidopsis provide mechanical protection against abrasion, wounding and insect herbivory, protect from water loss, light and temperature stress as well as UV irradiation damage (Dalin *et al.,* 2008; Wagner *et al.,* 2004; Werker, 2000). They reduce wind velocity and maintain a highly water-saturated microenvironment, consequently limiting excessive transpiration through stomata. They might absorb water and nutrients as well as facilitate seed dispersal and pollen collection. Even dead trichomes may still serve a purpose in water absorption, seed dispersal and protection from abrasion. Being of the non-glandular type, the Arabidopsis trichomes are not presumed to secrete chemicals or signals negatively influencing insects or pathogens. Nonetheless, these trichomes have been demonstrated to express genes related to secondary metabolism and to synthesize secondary metabolites from a variety of classes (anthocyanins, flavonoids and glucosinolates) (Ebert *et al.,* 2010; Frerigmann *et al.,* 2012; Jakoby *et al.,* 2008; Mauricio and Rausher, 1997; Serna and Martin 2006; Walker *et al.,* 1999). Moreover, disease-related proteins like PR1, RPP5-like protein or TIR-NBS-LRR have been reported in trichomes of Arabidopsis (Bruner, 2009; Wienkoop *et al.*, 2004). Interestingly, evidence has been provided that high-level expression of an anti-fungal hydrolase in Arabidopsis trichomes using a transgenic approach confers an increased plant resistance to the phytopathogenic fungus *B. cinerea* (Calo *et al.,* 2006). It is thus hypothesized that apart from providing a physical barrier against abiotic damage and herbivory, *A. thaliana* trichomes might additionally be implicated in chemical-based defense and stress signaling responses against insects and pathogens.

Previously, the promoter of the Arabidopsis gene *At2g02360* encoding F-box-Nictaba has been demonstrated by GUS histochemical staining to be particularly active in the trichomes located on young rosette leaves (Chapter 3, Section 3.4.5). Also, eight MYB-like recognition sites and five T/G-box elements, which are *cis*-regulatory elements involved in trichome-specific gene regulation (Ni *et al.,* 2008; Shangguan *et al.,* 2008), have been found in the promoter sequence of *At2g02360* (Fig. A3.7), suggesting that this gene might be preferentially expressed in the trichomes. In order to investigate in more detail *F-box-Nictaba* gene expression in the trichomes, we have performed a qRT-PCR analysis on trichomes isolated from the leaves of *A. thaliana* plants of two ecotypes, i.e. Columbia-0 (Col-0) and Landsberg erecta-0 (Ler-0). This analysis clearly showed that *At2g02360* expression was indeed pronounced in the trichomes, particularly in those of Ler-0 background. This result was further supported by immunodetection experiments which revealed a much higher F-box-Nictaba content in the protein extracts from trichomes originating from *A. thaliana* Ler-0 plants. Furthermore, we studied the expression of genes encoding enzymes required for the synthesis of

Lewis A structures in Arabidopsis, i.e. β1,3-galactosyltransferase (GALT1) and α1,4-fucosyltransferase (FUT13). Lewis A motifs in plants have been identified either on the cell surface or on glycoproteins secreted by plant cells and as such are presumably involved in plant-pathogen interactions and in stress signaling (Fitchette *et al.,* 1999; Léonard *et al.*, 2002; Melo *et al.,* 1997). Gene expression analysis demonstrated that both GALT1 and FUT13 are highly up-regulated in the trichomes isolated from the leaves of *A. thaliana* Ler-0 plants, suggesting that carbohydrate motifs specifically recognized by F-box-Nictaba could also be abundant in these plant structures. These data confirm the pronounced occurrence of F-box-Nictaba in the trichomes and thus further support the hypothesis of its role in plant defense responses.

4.3 Materials and methods

4.3.1 Plant material

Seeds of WT *A. thaliana* ecotype Columbia-0 (Col-0) were purchased from Lehle Seeds (Round Rock, Texas, USA). Seeds of WT *A. thaliana* ecotype Landsberg erecta-0 (Ler-0) (NW20) and the mutant *GLABRA1* (N64) were obtained from the European Arabidopsis Stock Centre (NASC, University of Nottingham, UK). Arabidopsis seeds were sown in pot soil. To break dormancy, the sown seeds were first stratified at 4°C for 3 days in the dark. Afterwards, seeds were transferred to a controlled growth chamber (Conviron Germany GmbH, Berlin, Germany) set at 21°C with a 12/12 h light/dark photoperiod for seed germination and plant development. Rosette leaves were collected from 3-, 4- and 5-week-old plants (corresponding to developmental stages 1.10, 3.90 and 6.10, respectively, as defined by Boyes *et al.*, 2001). These leaves were either ground to a fine powder with a mortar and pestle and frozen at -80°C until further processing (RNA or protein extraction) or were immediately used for trichome isolation.

4.3.2 Trichome isolation

Leaf trichomes were isolated from rosette leaves according to Marks *et al.* (2008) with minor modifications. This method combines incubation of leaves in an EGTA-containing solution with vortexing in the presence of small glass beads (60–80 µm Ø) for fast and efficient isolation of intact trichomes. Extended incubation in EGTA weakens the connection between trichomes and surrounding epidermal cells via chelation of Ca^{2+} ions present in the pectins of the cell wall. As the attachment of the trichomes to leaves is already weakened by the action of EGTA, glass beads dislodge the trichomes protruding from the leaf surface, by bumping into them during intensive vortexing. Such isolated trichomes are then retrieved from the solution by filtering them out on a 100-µm cell strainer.

In short, approx. 2,5 g of rosette leaves were collected into a 50 ml BD Falcon tube containing 50 mg glass beads (60-80 µm Ø; Grace, Breda, The Netherlands) and 15 ml of a 1x EGTA/K-PBS solution (containing 50 mM EGTA, 100 mM KCl, 8 mM K_2HPO_4, 8 mM KH_2PO_4, pH 7.5). In total 10 tubes were prepared to isolate 1 batch of trichomes. All tubes were mixed using a Vortex machine at maximum speed (four cycles of 30 s mixing and 30 s rest on ice). Afterwards, solutions were filtered through a sieve (600 µm Ø) and collected in a beaker. Trichomes trapped in the plant material residing on the sieve were released by washing with 15 ml of 1x K-PBS solution (100 mM KCl, 8 mM K_2HPO_4, 8 mM KH_2PO_4, pH 7.5). The trichome solution was then filtered through a cell strainer (100 µm Ø, BD Falcon). Trichomes residing on the cell strainer were transferred to an 1.5 ml eppendorf tube using 1x K-PBS. Finally, the solution was centrifuged at 150 g for 1.5 min at 4°C and the supernatant was carefully removed. Purified trichomes were immediately frozen in liquid nitrogen and stored in -80°C until further processing. Yield, purity and integrity of the isolated trichomes was assessed by transmission microscopy using a Leica DFC400 microscope (Leica) using the Leica Application Suite software packages.

4.3.3 Total RNA extraction

For total RNA extraction, 1 ml TRI Reagent (Sigma-Aldrich) and 50 mg glass beads (300 µm Ø, Sigma-Aldrich) were added to approx. 60,000 – 80,000 trichomes (isolated from 25 g of rosette leaves). The solution was vortexed at max speed (five cycles of 20 s mixing and 20 s rest on ice). For RNA extraction from unprocessed rosette leaves, 1 ml TRI Reagent was added to approx. 100 mg samples previously ground to a fine powder with a mortar and pestle. Trichome and complete leaf tissue extracts were then processed according to the manufacturer's recommendations.

4.3.4 cDNA synthesis and RT-PCR analysis

DNase treatment of extracted RNA, cDNA synthesis and RT-PCR were performed as described in Chapter 3, Section 3.3.5, 'RNA extraction, cDNA synthesis and RT-PCR analysis'.

4.3.5 qRT-PCR analysis

qRT-PCR analysis was performed as described in Chapter 3, Section 3.3.6, 'Quantitative RT-PCR (qRT-PCR) analysis'.

4.3.6 Protein extraction

For protein extraction, approx. 300,000 trichomes were re-suspended in 200 µl of 50 mM Tris–HCl (pH 7.5) and mixed together with approx. 150 mg of 300 µm glass beads at max speed (five cycles of 20 s mixing and 20 s rest on ice). The resulting suspension was then centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatant was collected. Another 200 µl of 50 mM Tris–HCl (pH 7.5) was added to the remaining pellet and the suspension was again vortexed and centrifuged. The resulting supernatant was collected as described above. All supernatants together constituted the final trichome protein extract.
Protein from rosette leaves was extracted by grinding leaves to a fine powder with a mortar and pestle, adding 1 ml of 50 mM Tris–HCl (pH 7.5) to approx. 100 mg of powdered samples and grinding them a second time. The resulting crude extract was transferred to a 1.5 ml eppendorf tube and centrifuged at 12,000 rpm for 10 min at 4°C. The protein concentration of all samples was determined using the Nanodrop 2000 Spectrophotometer (Thermo Scientific).

4.3.7 Western blot analysis

10 µg of total protein extracts were analyzed by SDS-PAGE in gradient (4-15%) Mini-PROTEAN®TGX™ precast polyacrylamide gels (Biorad) under reducing conditions (Laemmli, 1970) and subsequent Western blot analysis. After transfer of the proteins onto a 0.45 µm PVDF membrane (BiotraceTM PVDF, PALL), the membrane was blocked with 5% (w/v) BSA in Tris-buffered saline for 1 h. The Arabidopsis F-box-Nictaba protein was then detected by incubation for 1 h incubation with a specific primary guinea pig antibody (1/1,000) (see Chapter 3, Section 3.3.11), followed by 1 h with a secondary HRP-coupled goat anti-guinea pig IgG antibody (1/15,000) (Invitrogen) and a final 1 h incubation with PAP (1/2,000) (Sigma). The PageRuler prestained protein ladder (Thermo Scientific) was marked with the WesternBright Chemipen (Isogen Life Science, Belgium). Visualization of the immunoreactive proteins was performed by ECL chemiluminescence using the Pierce ECL Western blotting Substrate kit (Thermo Scientific) and a ChemiDoc MP imaging system (Biorad). After immunodetection the proteins on the blot were visualized by blot staining with Coomassie Brilliant Blue R-250.

4.4 Results

4.4.1 Isolation of pure and intact trichomes

To isolate trichomes from rosette leaves of WT *A. thaliana* Col-0, a procedure developed by Marks *et al.* (2008) was applied. The method relies on chelation of Ca^{2+} ions present in cell wall pectins,

leading to a weakened connection between trichomes and surrounding epidermal cells, resulting in efficient detachment of intact and enzymatically active trichomes. To evaluate if this method indeed allows to retrieve trichomes which are still biologically active, trichomes isolated from the leaves of transgenic *p35SCaMV:GUS* Arabidopsis plants were subjected to a GUS histochemical assay. Since as is presented in Fig. 4.1A these trichomes are stained blue, it could be concluded that they maintained their enzymatic activity after purification. In order to get enough trichome material from WT plants for subsequent qRT-PCR and immunodetection experiments, the isolation procedure was scaled up according to the expected trichome yields reported by Marks *et al.* (2008).

As estimated by counting with a hemocytometer, single trichome isolations from rosette leaves of WT *A. thaliana* Col-0 gave satisfactory yields in the range of 60,000 – 80,000 trichomes from approx. 25 g of leaves. Judging by microscopic analysis (Fig. 4.1B), the isolated trichomes were pure, undamaged and thus suitable for subsequent analyses. Depending on the sample, isolated trichomes allowed to extract 1.0-5.0 µg of total RNA and 30-40 µg of total protein.

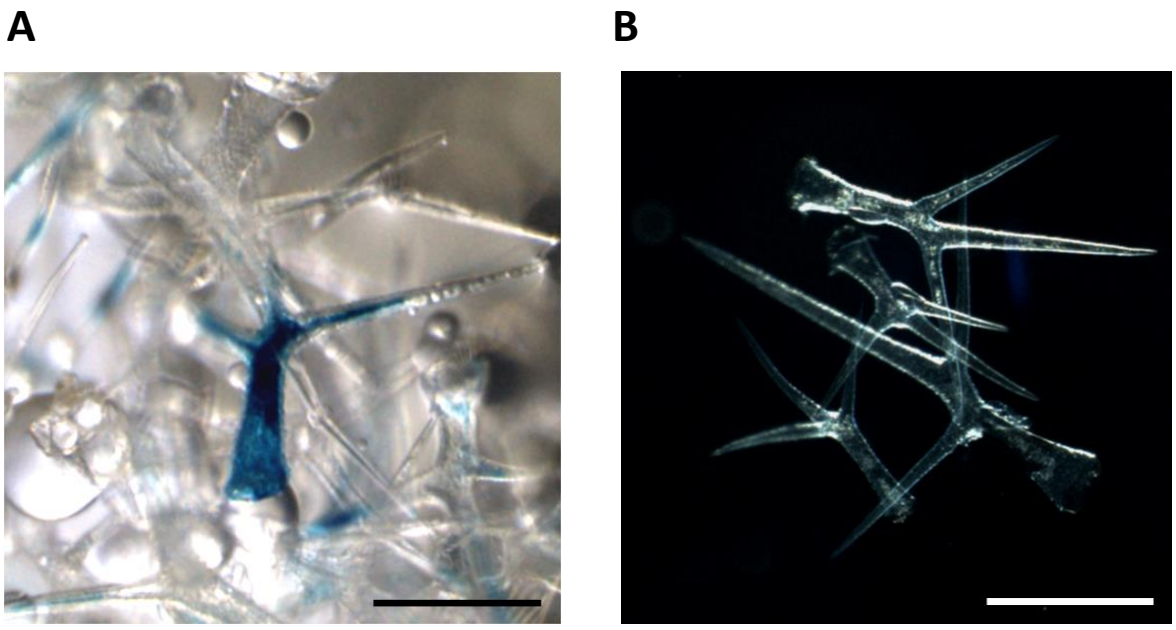


Fig. 4.1 Microscopic images of isolated trichomes visualized under transmission light. A, Trichomes isolated from the rosette leaves of transgenic *p35SCaMV:GUS* Arabidopsis plants subjected to a GUS histochemical assay after purification. B, Trichomes isolated from the rosette leaves of 4-week-old WT *A. thaliana* Col-0 plants. Scale bars represent 100 µm.

4.4.2 Gene expression analysis

4.4.2.1 *F-box-Nictaba* is predominantly expressed in the trichomes of *A. thaliana* plants

Given the apparent activity of the *pAt2g02360:GUS* reporter construct in trichomes present on the shoot meristem, first leaves and young rosette leaves in the transgenic *A. thaliana* GUS lines (see Chapter 3, Section 3.4.5), the expression of the *F-box-Nictaba* gene was quantified by qRT-PCR in trichomes purified from WT *A. thaliana* Col-0 rosette leaves originating from 3-, 4- and 5-week-old

plants (corresponding to developmental stages 1.10, 3.90 and 6.10, respectively, as defined by Boyes *et al.* 2001). The *At2g02360* mRNA levels measured in trichomes were slightly higher than those measured in unprocessed rosette leaves (containing trichomes) (Fig. 4.2A). At all tested time points the *F-box-Nictaba* expression was increased in the trichomes up to approximately 1.5-fold in 4-week-old plants and 2-fold in 3- and 5-week-old plants.

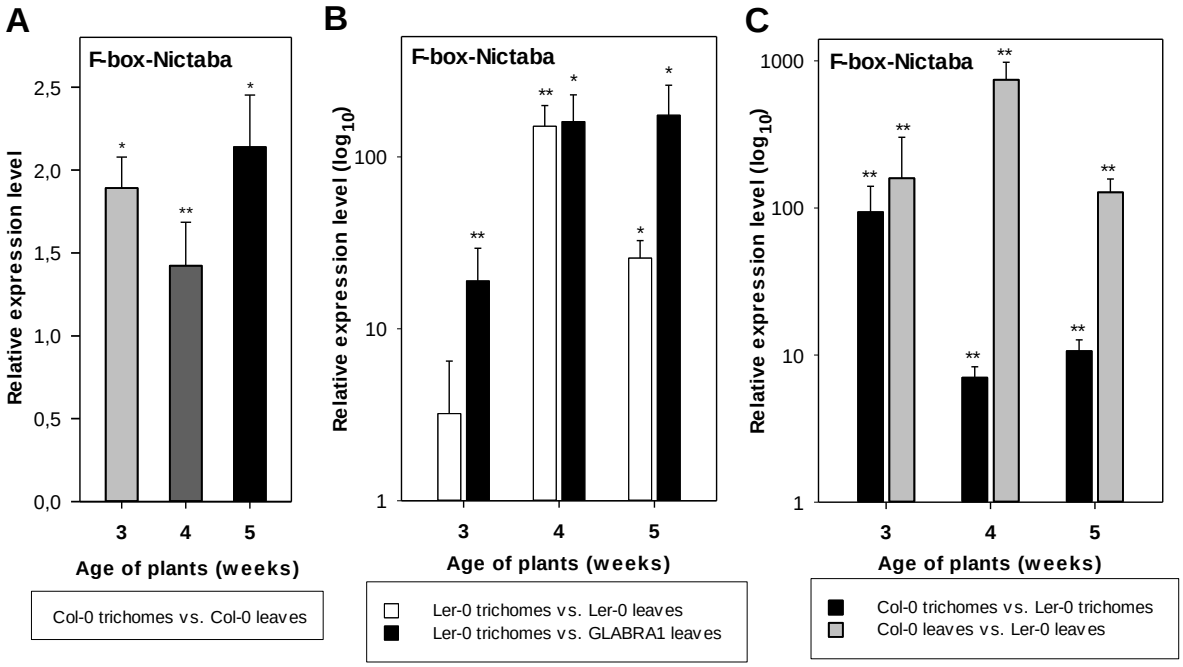


Fig. 4.2 Relative transcript levels of *At2g02360* in the trichomes isolated from rosette leaves of WT Arabidopsis Col-0 and Ler-0 plants compared to the corresponding gene expression levels in unprocessed rosette leaves from WT Col-0, WT Ler-0 and *GLABRA1* Arabidopsis plants measured in 3-, 4- and 5-week-old plants. Relative transcript levels were determined by qRT-PCR analyses of two independent biological experiments. *n*=2; error bars ± SE (*p<0.05; **p<0.01). Asterisks indicate statistically significant differential expression compared to control samples (unprocessed rosette leaf material). A, Relative gene expression levels in the trichomes of Col-0 background compared to unprocessed rosette leaves of Col-0 background. B, Relative gene expression levels in the trichomes of Ler-0 background compared to unprocessed rosette leaves of Ler-0 background (white bars) or compared to unprocessed rosette leaves of *GLABRA1* Arabidopsis plants (black bars). C, Comparison of gene expression levels of *F-box-Nictaba* in the trichomes (black bars) and in unprocessed rosette leaves (gray bars) of WT Col-0 versus WT Ler-0 Arabidopsis plants.

F-box-Nictaba gene expression was also analyzed in rosette leaves of the mutant *A. thaliana* plants completely lacking trichomes. These so-called *GLABRA1* plants are impaired in the *GL1* gene (*At3g27920*) which encodes a MYB-like transcription factor required for trichome development (Oppenheimer *et al.*, 1991). Since the *GLABRA1* mutants are made in a Landsberg erecta-0 (Ler-0) background, *F-box-Nictaba* expression was also examined in rosette leaves and in trichomes isolated from rosette leaves originating from WT *A. thaliana* Ler-0 plants. Trichome purification from rosette leaves of Ler-0 plants yielded similar amounts of trichomes as for Col-0 plants. However, trichomes of Ler-0 plants seemed to be smaller than trichomes present on Col-0 rosette leaves of the same age

(results not shown). In comparison to Col-0 plants, in Ler-0 background the relative expression of the *F-box-Nictaba* gene was much more pronounced in trichomes with an up-regulation of *F-box-Nictaba* mRNA levels of 3.2-fold (although not statistically significant), 150-fold and 25-fold compared to total leaf tissue from 3-, 4- and 5-week-old plants, respectively (Fig. 4.2B, white bars). When the *At2g02360* transcript levels were compared in Ler-0 trichomes to these levels in the trichomeless *GLABRA1* rosette leaves, 20-, 160- and 175-fold higher expression of *At2g02360* was observed in trichomes from 3-, 4- and 5-week-old Ler-0 plants, respectively (black bars).

However, the absolute transcript levels for the *F-box-Nictaba* gene were higher in WT *A. thaliana* leaves of plants with a Col-0 background compared to leaves from WT plants with a Ler-0 background, at all developmental stages investigated, irrespective whether the isolated trichomes or complete rosette leaves of both ecotypes were assessed (Fig. 4.2C). The differential expression was most striking when unprocessed rosette leaves were compared (gray bars): *At2g02360* mRNA levels were approx. 160, 740 and 130 times higher in leaves of 3-, 4- and 5-week-old plants of Col-0 ecotype, respectively, compared to leaves of the same age from plants with a Ler-0 background. In the trichomes isolated from 3-, 4- and 5-week-old WT *A. thaliana* Col-0 plants *F-box-Nictaba* gene expression was 90-, 7- and 10-fold higher than in the trichomes of 3-, 4- and 5-week-old plants from WT *A. thaliana* Ler-0, respectively (Fig. 4.2C, black bars).

4.4.2.2 The *GALT1* and *FUT13* genes are up-regulated in the trichomes of WT *A. thaliana* Ler-0 plants but not in the trichomes of WT *A. thaliana* Col-0 plants

qRT-PCR analysis was also performed for two genes encoding the enzymes indispensable for Lewis A epitope synthesis in *A. thaliana*: *i.e. At1g26810* for β1,3-galactosyltransferase (GALT1) expression and *At1g71990* for α1,4-fucosyltransferase (FUT13) expression (Fig. 4.3) (Léonard *et al.*, 2002; Strasser *et al.*, 2007).

According to Fig. 4.3A, the transcript levels for *GALT1* were significantly lower in the trichomes of WT *A. thaliana* Col-0 plants compared to those measured in the unprocessed rosette leaves of the same plants at all investigated developmental stages, the lowest value being a 5-fold decrease of *GALT1* expression in trichomes of 4-week-old plants. In 3- and 5-week-old plants *GALT1* mRNA levels were approximately 2 and 3 times lower in trichomes than in rosette leaves, respectively. *FUT13* did not show a significant differential expression between trichomes and unprocessed rosette leaves at any of the investigated developmental stages (Fig. 4.3B).

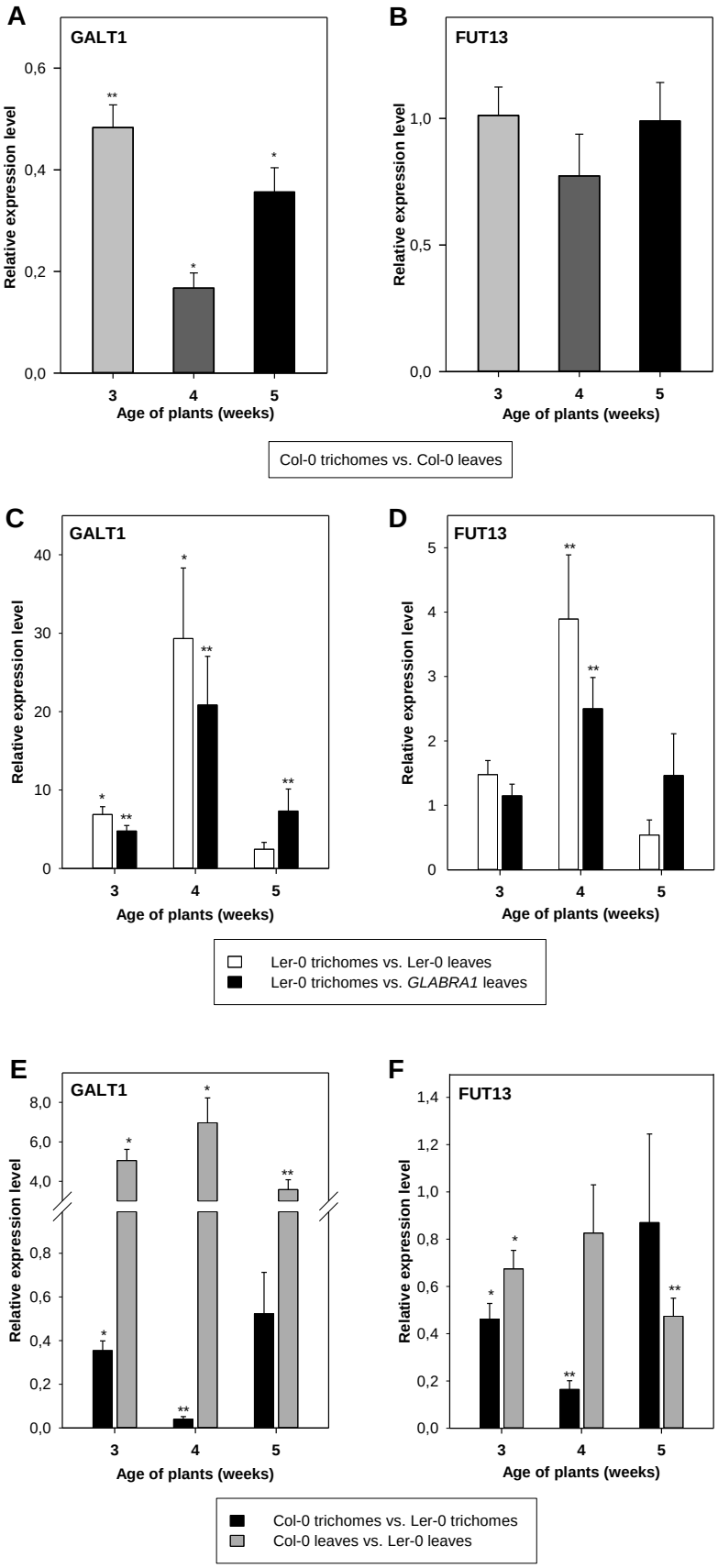


Fig. 4.3 Relative transcript levels of *GALT1* (encoded by *At1g26810*) and *FUT13* (encoded by *At1g71990*) in the trichomes isolated from rosette leaves of WT Col-0 and WT Ler-0 Arabidopsis plants vs. corresponding gene expression levels in unprocessed rosette leaves from WT Col-0, WT Ler-0 and *GLABRA1* Arabidopsis plants measured in 3-, 4- and 5-week-old plants. Relative transcript levels were determined by qRT-PCR analyses in two independent biological experiments. *n*=2; error bars ± SE. Asterisks indicate statistically significant differential expression compared to control samples (unprocessed rosette leaf material) (*p<0.05; **p<0.01). A-B, Relative gene expression levels of *GALT1* (A) and *FUT13* (B) in the trichomes of WT Col-0 Arabidopsis plants compared to unprocessed rosette leaves from WT Col-0 Arabidopsis plants. C-D, Relative gene expression levels of *GALT1* (C) and *FUT13* (D) in the trichomes of WT Ler-0 Arabidopsis plants versus unprocessed rosette leaves of WT Ler-0 Arabidopsis plants or versus unprocessed rosette leaves of *GLABRA1* Arabidopsis plants. E-F, Comparison of gene expression levels of *GALT1* (E) and *FUT13* (F) in the trichomes and in unprocessed rosette leaves of WT Col-0 vs. WT Ler-0 Arabidopsis plants.

In contrast, in trichomes of 3- and 4-week-old WT *A. thaliana* Ler-0 plants, the expression of the *GALT1* gene was markedly up-regulated by 7- and 29-fold, respectively, when compared to its levels in unprocessed rosette leaves originating from these plants (Fig. 4.3C, white bars). Comparison of the *GALT1* expression levels in trichomes of WT *A. thaliana* Ler-0 plants to those in the trichomeless *GLABRA1* leaves revealed that *GALT1* mRNA levels are significantly higher in the trichomes by 5-, 20- and 7-fold, respectively, in the three plant stages tested (black bars). The differences in *FUT13* expression levels between trichomes and unprocessed rosette leaves from WT *A. thaliana* Ler-0 plants were smaller than for the *GALT1* gene expression. As shown in Fig. 5D, *FUT13* expression in trichomes of 4-week-old plants was only 4 times higher, whereas in 3- and 5-week-old plants there was no apparent difference in *FUT13* expression in trichome RNA and total RNA of Ler-0 leaf material (white bars). When *FUT13* expression was compared in WT Ler-0 plants and *GLABRA1* (Ler-0) plants the results showed a significant up-regulation by 2.5-fold for *FUT13* expression in trichomes of Ler-0 plants compared to *GLABRA1* leaves of 4-week-old plants (black bars).

Interestingly, the up-regulation of the two glycosyltransferases in the Ler-0 trichomes was lower when compared to the trichomeless *GLABRA1* rosette leaves (black bars) than when compared to the complete Ler-0 rosette leaves (white bars; 3-week- and 4-week-old plants in Fig.4.3C, as well as 4-week-old plants in Fig.4.3D). Only when the *GALT1* transcript levels were assessed in 5-week-old plants, the up-regulation value in the trichomes was higher when compared to the trichomeless *GLABRA1* rosette leaves (Fig.4.3C). It has to be noted here that even though the trichomeless *GLABRA1* plants derive originally from a Ler-0 background (and thus were chosen to measure trichome-specific expression in a Ler-0 background) they are not a perfect control. Due to the mutation in *gl1*, they do not only lack trichomes, they are also more susceptible to stress (Xia *et al.,* 2010). Therefore, it could be that *GALT1* gene expression in *GLABRA1* plants is changed in comparison to the WT Ler-0 plants. Indeed, apparently, the *GALT1* expression in the leaves of *GLABRA1* plants was higher at indicated time points than in the leaves of Ler-0 plants. Still, this does not compromise the result obtained after comparison of *GALT1* and *FUT13* expression in Ler-0 trichomes compared to complete Ler-0 leaves, showing significant up-regulation in the trichomes.

A comparative analysis of *GALT1* gene expression in WT *A. thaliana* plants of different ecotypes showed that the *GALT1* gene was 3.5-7 times more expressed in the leaves of Col-0 plants compared to the corresponding leaves of Ler-0 plants (Fig. 4.3E, gray bars), with the highest differential expression in 4-week-old plants. In contrast, *GALT1* gene expression was 3-fold and 30-fold higher in the trichomes of 3- and 4-week-old Ler-0 plants than in the corresponding leaves of Col-0 plants (Fig. 4.3E, black bars). On the other hand, WT *A. thaliana* Ler-0 plants in general had slightly higher transcript levels for *FUT13* than the WT *A. thaliana* Col-0 plants in unprocessed rosette leaves (Fig. 4.3F, gray bars). More important differences were observed in the trichomes of 3- and 4-week-old plants, where the *FUT13* transcript level was 2- and 5-fold higher in the Ler-0 background (4.3F, black bars).

4.4.3 F-box-Nictaba protein occurrence in trichomes

As already described previously (Chapter 1), F-box-Nictaba protein is a member of the family of nucleocytoplasmic plant lectins. Since these types of lectins are presumed to be present in the nucleus and cytoplasm of plant cells in minute concentrations (Lannoo and Van Damme, 2010), they are rarely detectable by immunodetection methods. Nevertheless, based on the qRT-PCR results indicating very high *F-box-Nictaba* gene up-regulation in the trichomes of *A. thaliana* plants (especially of ecotype Ler-0), Western blot analysis was performed on total protein extracts from unprocessed leaf material and from isolated trichomes of 4-week-old WT Col-0 and WT Ler-0 *A. thaliana* plants as well as from unprocessed leaf material from the leaves of mutant *GLABRA1* plants using a specific anti-F-box-Nictaba antibody. As depicted in Fig. 4.4, two distinct bands could be detected in the protein extracts from trichomes of both ecotypes, although the intensity of the signals for the trichome extract from Ler-0 was much stronger. Interestingly, the two polypeptides have a much higher MW than expected for F-box-Nictaba (31.3 kDa). MW estimation using calibration of the protein marker, yielded polypeptides of approximately 61.4 kDa and 68.1 kDa. No bands were detected for any of the protein extracts from unprocessed leaves.

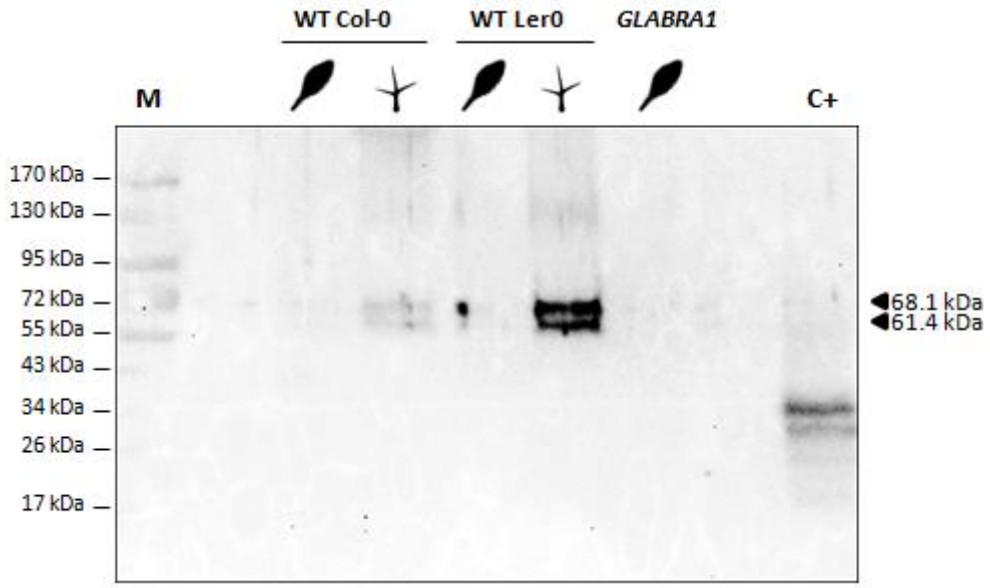


Fig. 4.4 Western blot on total protein extracts purified from unprocessed rosette leaves and trichomes of 4-week-old WT Col-0 and WT Ler-0 *A. thaliana* plants as well as from unprocessed rosette leaves of the mutant *GLABRA1* plants. Immunodetection was performed using a specific anti- F-box-Nictaba antibody. Equal amounts of proteins (10 µg) were loaded in each lane. M: protein marker; C+: positive control (0.2 µg of purified recombinant Nictaba domain of F-box-Nictaba protein).

4.5 Discussion

4.5.1 *F-box-Nictaba* expression is pronounced in non-glandular *A. thaliana* trichomes

F-box-Nictaba gene expression was quantified in trichomes isolated from rosette leaves of 3-, 4- and 5-week-old WT *A. thaliana* Col-0 plants (Fig. 4.2A). The relative expression level of *At2g02360* in trichomes was calculated in comparison to *At2g02360* mRNA levels in unprocessed rosette leaves (still comprising trichomes). Intriguingly, despite a clear *F-box-Nictaba* promoter activity in the trichomes, only an approximately 2-fold up-regulation of *F-box-Nictaba* was detected in the trichomes of Col-0 plants. This discrepancy between the GUS staining data and the gene expression analysis via qRT-PCR is very likely due to the trichomes still present on the rosette leaves taken as a control sample, leading to a considerable underestimation of the expression value. Ideally, gene expression in the isolated trichomes should be assessed relatively to rosette leaves devoid of trichomes to minimize this effect, but unfortunately extensive tissue damage after trichome isolation does not allow reliable RNA isolation from the processed leaf material. To address this issue, *F-box-Nictaba* transcript levels were analyzed in rosette leaves of *GLABRA1* mutant *A. thaliana* plants, which are completely trichomeless (Oppenheimer *et al.*, 1991). Since those mutant plants were created in a Ler-0 background, the expression analysis required trichome isolation from rosette leaves of WT *A. thaliana* Ler-0 plants. Unexpectedly, in contrast to the outcome of the expression analysis in Col-0 plants, the trichome-specific expression of *F-box-Nictaba* gene was already clear in Ler-0 background when compared to unprocessed rosette leaves, with a higher gene expression in trichomes up to 150 times (Fig. 4.2B). Comparison of *F-box-Nictaba* expression in Ler-0 trichomes compared to trichomeless *GLABRA1* rosette leaves showed an even more striking up-regulation in trichomes up to 175-fold increase in transcript levels. Therefore, as presumed before based on the promoter sequence analysis, most probably *F-box-Nictaba* gene expression in the trichomes is under control of MYB transcription factors (Dubos *et al.,* 2010) which can bind to the multiple MYB-like *cis*-regulatory elements present in the F-box-Nictaba promoter (Fig. A3.1; Ni *et al.,* 2008; Shangguan *et al.,* 2008).

One of the reasons why it was not possible to demonstrate a trichome-specific *F-box-Nictaba* expression in Col-0 plants by qRT-PCR, while it was perfectly feasible in Ler-0 plants even when the relative expression was measured compared to unprocessed rosette leaves, is the difference in trichome density between the two ecotypes. As reported by Larkin *et al.* (1996) and Symonds *et al.* (2011), Col-0 plants contain approximately 3.5 times more trichomes on their leaves (and slightly bigger ones) than Ler-0 plants, and therefore the underestimation of *F-box-Nictaba* expression in trichomes of Col-0 when compared to unprocessed rosette leaves is much more critical. Another possible explanation is brought forward by comparison of the absolute transcript levels for the *F-box-Nictaba* gene between WT *A. thaliana* plants with a Col-0 background to WT plants with a Ler-0 background. As depicted in Fig. 4.2C, absolute transcript levels for the *F-box-Nictaba* gene were higher in Col-0 than in Ler-0 background, irrespective whether transcript levels are compared in

isolated trichomes or complete rosette leaves. This result can be confirmed by the microarray expression data available for natural variation between Arabidopsis ecotypes on the eFP browser (Winter *et al.*, 2007). Nevertheless, the differential expression between the two ecotypes was much more prominent when the unprocessed leaf materials were compared. However, despite the preferential expression of *F-box-Nictaba* in Col-0 trichomes as visualized by the GUS assay (Chapter 3, Section 3.4.5, Fig. 3.3B), some basal gene expression was also clear in non-trichome parts of the leaf (e.g. major leaf vein, Fig. 3.3B – 1.10 and 6.90b).

What is more, there were striking differences in the relative gene expression in the trichomes depending on the age of plants (especially between 3- and 4-week-old plants). The changes might be related to the prevalence of the trichomes during the development of Arabidopsis plants. Indeed, leaves produced early in rosette development lack trichomes on their abaxial side, whereas leaves produced later in time possess trichomes on both their adaxial and abaxial sides (Telfer *et al.,* 1997). Apparently, the timing of abaxial trichome formation is strongly affected by photoperiod conditions, where short day conditions delay the production of leaves with abaxial trichomes and, conversely, during long day photoperiod leaves with abaxial trichomes develop faster. Interestingly, photoperiod sensitivity of abaxial trichome formation develops gradually over time and exhibits its highest level about 24 days after germination (Chien and Sussex, 1996). This timing would correspond well with the striking differences observed in the experiments here in the relative gene expression levels in the trichomes especially between 3- and 4-week-old plants.

4.5.2 The genes *GALT1* and *FUT13* encoding enzymes required for the synthesis of glycan structures specifically recognized by F-box-Nictaba are co-expressed in *A. thaliana* trichomes of Ler-0 background

Previously, the Arabidopsis F-box-Nictaba protein has been shown to be a functional lectin that can bind *N*- and *O*-glycans containing LacNAc structures, Lewis A, Lewis X, Lewis Y and type-1 B antigen motifs (Stefanowicz *et al.*, 2012). As stated in Chapter 2, LacNAc-containing glycans have only been discovered in higher animals, parasitic nematodes, viruses and certain pathogenic bacteria in which they are present on cell surface glycoproteins and glycolipids (Zhou, 2003; Stanley and Cummings, 2009; van Die and Cummings 2010). At present, plants (including mosses and ferns as well as seed plants) were only reported to contain Lewis A-modified *N*-glycans. Other LacNAc structures have not been identified in plants yet. Since *N*-glycan modification with Lewis A motifs takes place in the Golgi apparatus, plant-specific Lewis epitopes were only shown on some extracellular glycoproteins and on membrane-bound glycoproteins present at the plant cell surface (Melo *et al.*, 1997; Fitchette *et al.,* 1999; Léonard *et al.*, 2002; Koprivova *et al.*, 2003; Viëtor *et al.*, 2003; Parsons *et al.*, 2012). Yet, it is not clear if and how the Arabidopsis F-box-Nictaba protein, which is localized in the nucleus and cytoplasm of the plant cell, can bind to these plant secreted or membrane-bound Lewis A structures. Moreover, in contrast to most plant families (Fitchette-Lainé *et al.*, 1997), the occurrence of Lewis A

motifs in Arabidopsis was shown to be generally low and tissue specific with highest levels in pedicels, stems and nodes, moderate levels in siliques and shoot apex, but was not detectable in the leaves (Strasser *et al.* 2007). Nevertheless, to our knowledge, Lewis A appearance has not been studied yet in *A. thaliana* trichomes. Thus, since F-box-Nictaba expression is prominent in the trichomes, we addressed the important question whether Lewis A motifs could be specifically synthesized in the trichomes as well. Analysis of the expression of the genes encoding a β1,3-galactosyltransferase (GALT1) and α1,4-fucosyltransferase (FUT13), the two enzymes indispensable for Lewis A synthesis in *A. thaliana* (Léonard *et al.,* 2002; Strasser *et al.,* 2007), demonstrated that both of them are indeed expressed in trichomes of *A. thaliana* plants. However, expression analysis in the trichomes of Col-0 *A. thaliana* plants showed that *GALT1* mRNA levels were in fact significantly 2- to 5-fold lower in the trichomes of WT *A. thaliana* Col-0 plants compared to the complete leaf tissue (Fig. 4.3A). *FUT13* was not differentially expressed (Fig. 4.3B). Strikingly, both *GALT1* and *FUT13* were considerably up-regulated in the trichomes of WT *A. thaliana* with Ler-0 background reaching respectively up to 29-fold and 7-fold increase in transcript levels (Fig. 4.3C-D). This result strongly resembles the outcome of the *F-box-Nictaba* gene expression analysis in trichomes (Fig. 4.2A-B). What is more, all three genes reached their highest trichome-specific expression in 4-week-old Arabidopsis Ler-0 plants. These data indicate that the two enzymes required for biosynthesis of Lewis A structures and the carbohydrate-binding protein F-box-Nictaba are co-expressed in the trichomes of *A. thaliana* Ler-0 plants. Obviously, the fact that Lewis A-containing glycans could be synthesized in the trichomes does not indicate that they would be available to F-box-Nictaba within the nucleocytoplasmic compartment of the cell. Further studies are required to clarify the issue of the availability of specifically glycosylated proteins which could be recognized by F-box-Nictaba and targeted for proteasomal degradation.

4.5.3 The F-box-Nictaba protein is abundant in *A. thaliana* trichomes

The F-box-Nictaba protein was detectable in the trichomes isolated from rosette leaves of 4-week-old WT *A. thaliana* plants of both Col-0 and Ler-0 ecotypes, while no band for the protein could be visualized by Western blot analysis for any of the tested unprocessed leaf materials (Fig. 4.4). This result is in agreement with the data from the gene expression analysis by qRT-PCR and the previously obtained GUS histochemical staining results (Chapter 3, Section 3.4.5) showing preferential *At2g02360* gene expression and promoter activity in the trichomes. However, in contrast to the qRT-PCR data which showed 7-fold higher expression of the *F-box-Nictaba* gene in the trichomes of 4-week-old *A. thaliana* Col-0 plants than in the corresponding trichomes of Ler-0 background (Fig. 4.2C), F-box-Nictaba protein levels were much higher in the trichomes of Ler-0 plants. Yet, it has to be noted that as F-box proteins play a regulatory role in the cell by recognizing and targeting specific substrates for proteasomal degradation, likewise they are themselves tightly controlled by the UPS (de Bie and Ciechanover, 2011). It has been widely reported that F-box proteins are recognized by E3 Ub ligases, labeled with Ub, and immediately degraded by the proteasome (An *et al.,* 2010; Bashir *et*

al., 2004; Kim *et al.,* 2003; Klitzing *et al.,* 2011; Magori and Citovsky, 2011a; Wei *et al.,* 2004). With respect to rapid protein turnover, high mRNA levels do not necessarily mean that the corresponding F-box protein is abundantly present at a certain time point or location. What is more, it has been demonstrated that proteasomal degradation of F-box proteins can occur via the autoubiquitination mechanism in the absence of the specific substrate (Galan and Peter, 1999; Scaglione *et al.,* 2007; Yen and Elledge*,* 2008).

Interestingly, while the GALT1 and FUT13 genes required for Lewis A structures biosynthesis are highly up-regulated and co-expressed with the *F-box-Nictaba* gene in the trichomes of 4-week-old Ler-0 *A. thaliana* plants, their transcript levels were actually significantly (30-fold and 5-fold) lower in the trichomes of 4-week-old Col-0 plants compared to those in the trichomes of Ler-0 plants.

As shown in Fig. 4.4, instead of the expected single band of approximately 31.3 kDa, two distinct bands could be detected in both trichome extracts corresponding to 61.4 kDa and 68.1 kDa, which is roughly double of the expected protein MW. In fact, a very similar double-band pattern of F-box-Nictaba was previously observed in the protein extracts from the medium of *P. pastoris* cultures recombinantly expressing the His-tagged F-box-Nictaba protein (Chapter 2, Section 2.4.2, Fig 2.2A). Interestingly, for some cultures both the protein of expected size as well as the two distinct bands of higher MW were detectable (supplementary Fig. A4.1). What is more, transgenic *A. thaliana* plants transformed with a construct for overexpression of F-box-Nictaba protein also produced a protein of much higher MW than anticipated (Chapter 3, Section 3.4.7.2, Fig 3.10). The occurrence of two clearly separate protein bands is rather puzzling and could point towards protein modification as recently demonstrated for the SAI-LLP1 lectin-like glycoprotein (Armijo *et al.,* 2013). Based on the AA protein sequence, F-box-Nictaba comprises several putative sites for post-translational modifications including one *N*-glycosylation (Schwarz *et al.,* 2011; Strasser, 2014), six *O*-GlcNAcylation (Fitchette *et al.,* 2007), three *N*-myristoylation (Boisson *et al.,* 2003; Podell and Gribskov, 2004) and two SUMOylation sites (Park *et al.,* 2011) (Fig. 4.5). Until now it was demonstrated that the F-box-Nictaba protein recombinantly expressed as secreted protein in *P. pastoris* was indeed *N*-glycosylated and PNGaseF-driven removal of the glycan moiety resulted in a protein size shift by 2.6 to 3.8 kDa (supplementary Fig. A4.2). It has to be pointed out however, that although the sequence carries a putative *N*-glycosylation site, F-box-Nictaba is not predicted to follow the secretory pathway in *A. thaliana.* Clearly, further studies are needed to investigate the possibility of F-box-Nictaba post-translational modification(s) *in vivo*, especially in view of the putative relevance of these events for the functionality of F-box-Nictaba in the UPS and its physiological role in plants.

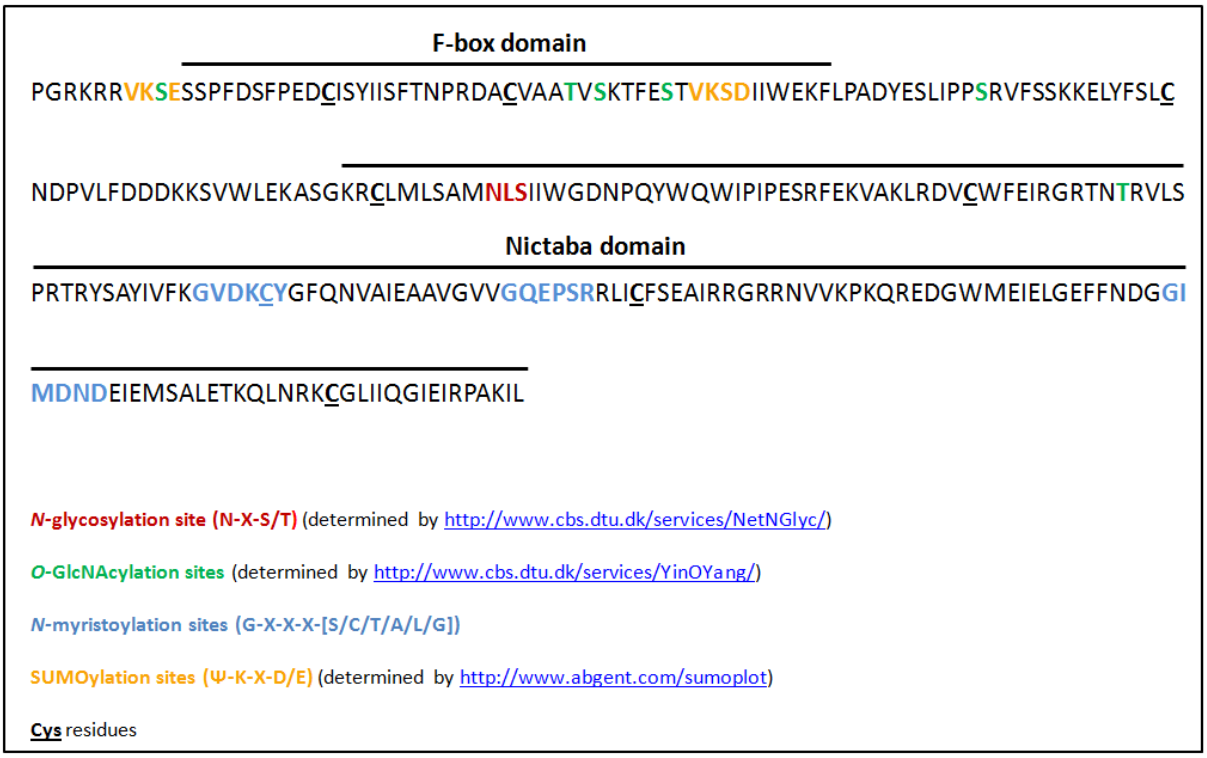


Fig. 4.5 Amino acid sequence of the F-box-Nictaba protein. Sites for putative post-translational modifications and location of Cys residues have been indicated on the sequence.

4.5.4 Conclusion

The outstanding over-expression of the carbohydrate-binding protein F-box-Nictaba in Arabidopsis trichomes of Ler-0 background in comparison to complete leaves, together with the fact that the two enzymes required for the biosynthesis of Lewis A structures are co-expressed in the trichomes of Ler-0 *A. thaliana* plants, is very intriguing and tempt to speculate regarding a possible interaction between F-box-Nictaba and these glycan motifs. Obviously, the fact that Lewis A-containing glycans are synthesized in the trichomes does not indicate that they are available to F-box-Nictaba within the nucleocytoplasmic compartment of the cell. Further studies are required to clarify the issue of the availability of specifically glycosylated proteins which could be recognized by F-box-Nictaba and targeted for proteasomal degradation. Our results also show that one has to be extremely careful when drawing conclusions based on biological data available for the same plant species but of different genetic background, as differences may be tremendous. Finally, by demonstrating a remarkable over-expression of a stress-related glycan-binding F-box protein and co-expression of the two enzymes required for Lewis A structure biosynthesis in Arabidopsis trichomes, our research sheds a new light on the putative role of the non-glandular trichomes in plant stress responses and glycan signaling.

Chapter 5

General discussion and perspectives

Plants are constantly challenged with a plethora of different environmental stresses and as sessile organisms they cannot avoid these threats. Consequently, both abiotic and biotic stresses tremendously affect the productivity of crop plants and constitute a major issue of contemporary agriculture and food security worldwide (Godfray *et al.,* 2010; Maxmen, 2013; Oerke, 2006). Nevertheless, plants have developed diverse defense mechanisms which enable survival under unfavorable conditions. Studies of these natural strategies are necessary in order to understand the complexity of plant stress responses and to implement the acquired knowledge in development of more resistant, high yield and improved quality crops (Agarwal *et al.,* 2013; Bita and Gerats, 2013; Ferry and Gatehouse, 2010; Jewell *et al.,* 2010; Reguera *et al.,* 2012).

One of the many different plant strategies to respond to environmental cues involves a specific group of stress-inducible carbohydrate-binding proteins, called lectins. These specialized proteins are presumably playing a role in stress by mediating glycan signaling (Lannoo and Van Damme, 2010; Van Damme *et al.,* 2011), however their modes of action are largely unclear. Concurrently, in view of the exceptional complexity, glycosylation becomes recognized as a multidimensional coding system with important, yet not well studied physiological functions (Rüdiger and Gabius, 2009; Pilobello and Mahal, 2007). Another plant stress defense mechanism, which has attracted growing attention of numerous reasearch groups in the last years, is the UPS. It is a multifarious machinery responsible for the elimination of misfolded or damaged proteins but also playing a pivotal role in controlling a multitude of physiological processes by selective degradation of key regulatory proteins. Particularly in plants, this highly complex and tightly regulated system appears to be of crucial importance since in no other kingdom the UPS comprises as many remarkably diverse components (Vierstra, 2009). The key constituents are the F-box proteins conferring specificity to the UPS by selective recognition and interaction with the targets destined for degradation. To date, more than 800 genes encoding putative members of the F-box protein family have been found in *A. thaliana* (Hua *et al.,* 2011), all of which could theoretically target different substrates for proteasomal degradation. Thereby, F-box proteins regulate a miriad of cellular events and allow fast as well as highly specific plant responses to internal and external signals (Guo *et al.,* 2013; Kelley and Estelle 2012; Lechner *et al.,* 2006; Marino *et al.,* 2012).

The function of most of the F-box proteins and their relevance for plant physiology remain very vague. In particular, little is known regarding the role of plant F-box proteins in glycan signaling. A few years ago, a group of putative plant-specific glycan-binding F-box proteins have been identified (Delporte *et al.,* 2015; Dinant *et al.,*2003; Lannoo *et al.,* 2008), which could presumably function in Ub-mediated glycoprotein degradation, and thus integrating both the UPS field and the glycosylation system as a mechanism in plant stress signaling. The members of this protein family contain a C-terminal domain homologous to Nictaba, the nucleocytoplasmic lectin from tobacco plants (Chen *et al.,* 2002; Lannoo, 2007), and are widespread in the plant kingdom (Delporte *et al.,* 2015; Lannoo *et al.,* 2008).

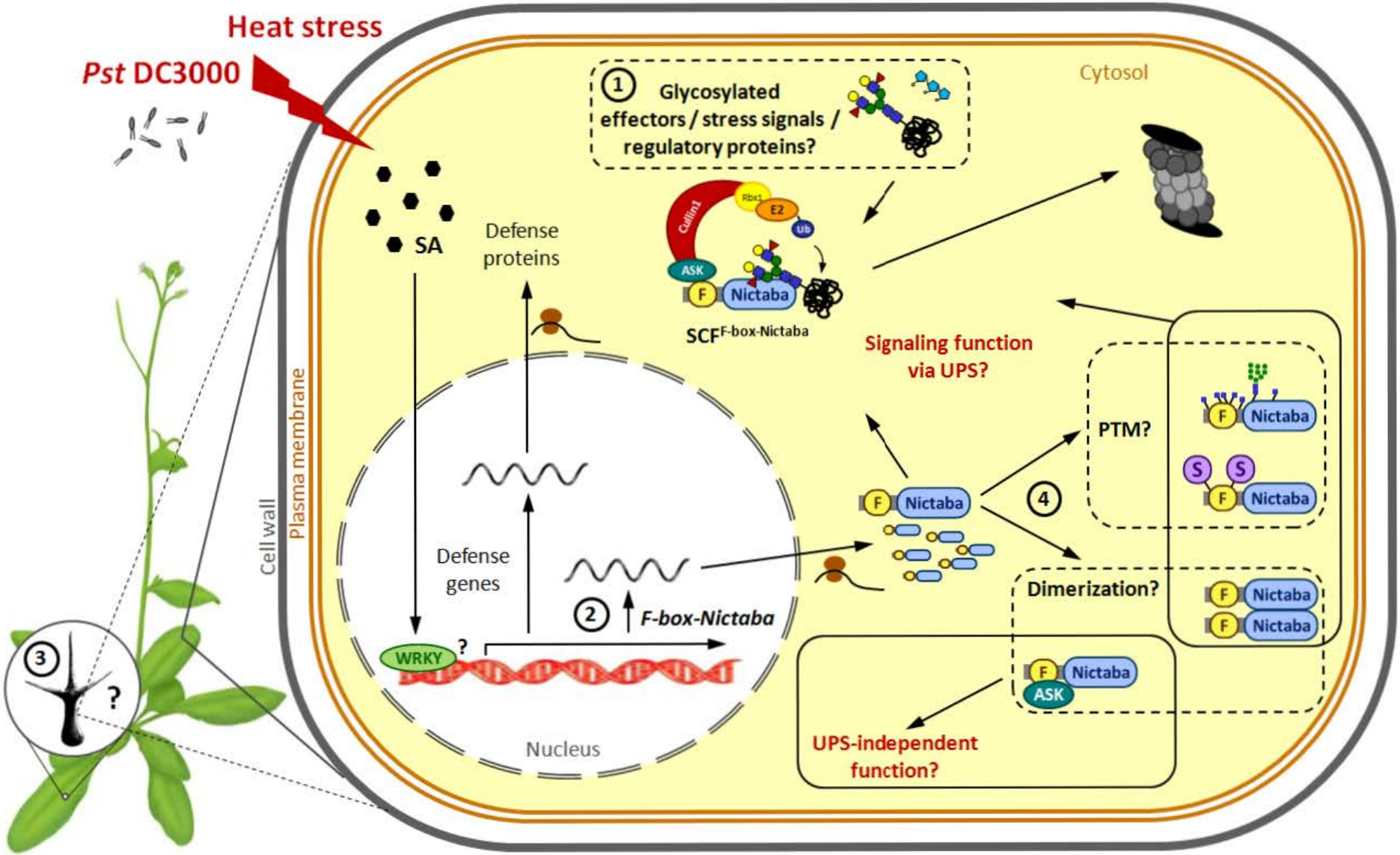


Fig. 5.1 Hypothetical model representing the physiological processes F-box-Nictaba might be involved in in *A. thaliana* cells. Numbers correspond to the sections in this Chapter.

This PhD work was dedicated to study the physiological relevance of one of these F-box-Nictaba homologs from *A. thaliana,* showing the highest sequence similarity with the Nictaba protein from tobacco. Using glycan array technology, detailed expression analyses and experiments on transgenic plants, the involvement of F-box-Nictaba in plant physiology and stress responses was investigated. This chapter reflects on the significance of the resulting findings, prospects for future reasearch and, in broader perspective, how these studies could contribute to bioengineering of more resistant plants. Fig. 5.1 presents an overview of processes in which F-box-Nictaba is or might be involved as discussed further.

5.1 Interaction of F-box-Nictaba with glycans

By means of glycan array technology we have demonstrated that the F-box-Nictaba protein, recombinantly expressed in *P. pastoris* and purified by affinity chromatography, is a functional lectin binding glycans through its C-terminal Nictaba-like domain (Chapter 2). Despite the initial hypothesis that, similarly to the tobacco Nictaba, F-box-Nictaba would preferentially interact with GlcNAc oligomers, the protein showed affinity towards substantially different glycan motifs. The only glycans both recognized by F-box-Nictaba and occuring in plants included type 1 LacNAc and Lewis A structures and feruloylated α1-5-L-arabinobiose/triose motifs. Obviously, such lectin specificity ruled out the suggested role of F-box-Nictaba in the Ub-mediated proteasomal degradation via the ERAD pathway of the misfolded or unassembled glycoproteins modified with a $Man_{3\text{-}9}GlcNAc_2$ *N*-glycan. Despite that, however, this distinct affinity for glycans does not preclude F-box-Nictaba from functioning in glycoprotein degradation via the UPS, but indicates that it will target another class of glycoproteins. In fact in mammals, where the first sugar-binding F-box proteins have been described, the members of the Fbs family differ in glycan binding specificities, but still all of them can associate with the components of the SCF Ub ligase complex, and thus presumably function in UPS (Glenn *et al.,* 2008). Likewise, the F-box-Nictaba protein under study has been demonstrated to interact with the Arabidopsis Skp1-like (ASK) constituents of the SCF in Arabidopsis (Arabidopsis Interactome Mapping Consortium, 2011; Takahashi *et al.,* 2004).

Still, a few questions arise in view of the potential function of F-box-Nictaba in Arabidopsis. First, it remains to be elucidated what is the function and significance of the recognition of these carbohydrate structures by F-box-Nictaba in *A. thaliana*. In order to answer this question the spatial distribution and possible interaction between F-box-Nictaba and its putative glycosylated ligands, i.e. glycoproteins containing type 1 LacNAc, Lewis A structures and/or feruloylated arabinans, needs to be investigated in more detail. Microscopical analyses have shown that F-box-Nictaba is located in the nucleocytoplasmic compartment of the plant cell (Lannoo, 2007). Unfortunately, little is known regarding carbohydrate structures present in the nucleus and cytoplasm (Funakoshi and Suzuki, 2009; Maeda and Kimura, 2014; Maeda *et al.,* 2010). At present, Lewis A epitopes have been reported only at the cell surface or in glycoproteins secreted by plant cells or in the Golgi apparatus

(Fitchette *et al.,* 1999; Léonard *et al.*, 2002; Melo *et al.,* 1997; Strasser *et al.,* 2007), while α1,5-arabinans occur exclusively in the cell wall (Pettolino *et al.,* 2012; Verhertbruggen *et al.,* 2009, 2013). Since apart from Lewis A in Golgi stacks, the recognized structures have not been detected within any intracellular compartment of the plant cell further experiments are needed to investigate the location of F-box-Nictaba protein and the presence of type 1 LacNAc structures and feruloylated α1,5-arabinans in plant tissues, plant cells and subcellular compartments.

Second, the function of the recognized carbohydrate structures in plant physiology is not understood well. Although the biological role of Lewis A epitopes in plants still remains unknown, in mammals they are involved in the cell-to-cell recognition, in selectin-dependent cell adhesion processes and in interactions with pathogens (Stanley and Cummings, 2009). Thus, by analogy, it could be hypothesized that Lewis A motifs present at the plant cell surface might also be involved in cell-to-cell communication or in plant-pathogen interactions. Moreover, secretion of glycoproteins containing Lewis A epitopes could suggest a putative role in stress signaling (Fitchette *et al.,* 1999; Léonard *et al.*, 2002; Melo *et al.,* 1997). Somewhat more information is available regarding the role of the arabinans in plants (Caffall and Mohnen, 2009; Harholt *et al.,* 2010). The feruloylated α1,5-arabinans have an important structural function in providing crosslinks between pectic polysaccharides via oxidative dimerization of feruloyl groups (Levigne *et al.,* 2004; Ralet *et al.,* 2005; Waldron *et al.,* 1997). Owing to their high mobility (Ha *et al.,* 2005), arabinans influence plant cell wall flexibility upon water deficit stress (Moore et al., 2008; Tang *et al.,* 1999). It has been demonstrated that a decreased content of arabinans stiffens the plant cell wall and affects plant responses to mechanical stress (Jones *et al.* 2003, 2005; Ulvskov *et al.*, 2005; Verhertbruggen *et al.,* 2013). The α1,5-arabinans are associated with fruit ripening in apple (Peña and Carpita, 2004) and are developmentally regulated in potato (Bush *et al.,* 2001). Feruloylated arabinans in the cell walls of guard cells turn out to be essential for stomatal movements, which become blocked after treatment with an endoarabinanase specific for α1,5-arabinan or with feruloyl esterase (Jones *et al.* 2003, 2005). Furthermore, α1,5-arabino-oligosaccharides are implicated in plant-pathogen interactions. The Arabidopsis *arad1* mutant with reduced arabinan content in the cell walls has been shown more susceptible to infection with the fungal pathogen *B. cinerea*, which is known to exploit a range of cell wall hydrolases as part of its invasion strategy (including the recently identified novel α-1,5-L-endoarabinanase; Nafisi *et al.,* 2014).

Thus, it appears that both Lewis A motifs and α1,5-arabinans are associated with plant defense responses and this could correspond well to the emerging role of F-box-Nictaba in plant-pathogen interactions described in Chapter 3. During the infection pathogens damage the plant cell wall using degradation enzymes (as the α-1,5-L-endoarabinanase from *B. cinerea*; Nafisi *et al.,* 2014), resulting in the release of damage-associated molecular patterns, which are the cell wall fragments serving as a signal for a plant to activate the intracellular defense machinery (Nühse, 2012; Wirthmueller *et al.,* 2013). Yet, these fragments do not enter the cell, but are perceived extracellularly by membrane-bound receptors. Nevertheless, plants can also synthesize numerous endogenous peptide signals in response to pathogen infection (Matsubayashi, 2014). They are usually produced as prepropeptides,

thus undergo postranslational proteolytic processing and modification including glycosylation. Actually, several of these peptides have been reported to be glycosylated with three residues of α-arabinose, however, they follow the secretory pathway. In contrast, the signaling plant peptide effectors localized in the cytosol, like the heat- and *Pst* DC3000-inducible kiss of death peptide activating programmed cell death, are not anticipated to be glycosylated (Blanvillain *et al.,* 2011; Huffaker *et al.,* 2006).

On the other hand, in view of the presumed role of F-box-Nictaba in plant defense against pathogens, putative targets could also be of foreign origin. Different glycans containing LacNAc and arabinan motifs have been found in the cell walls of mammalian bacteria and viruses and apparently are important for the virulence of these pathogens (Alderwick *et al.,* 2011; Mishra *et al.,* 2012; Monzavi-Karbassi *et al.,* 2004; Preston *et al.,* 1996; Wang *et al.,* 2000). Fucosylated lactosamines participate in adhesion of the HIV-1 envelope glycoprotein to dendritic cells (Monzavi-Karbassi *et al.,* 2004). Lactosamine motifs from lipooligosaccharides of Gram-negative bacteria are one of the main virulence factors allowing adhesion to mammalian cells (Preston *et al.,* 1996). The lipopolysaccharides of most *Helicobacter pylori* strains contain complex carbohydrates structurally related to the human blood group antigens (Lewis X, Y, A). Thereby, the bacteria exploit molecular mimicry of mammalian epitopes to avoid immune recognition for both persistent infection and pathogenesis (Wang *et al.,* 2000). Furthermore, cell walls of mycobacteria comprise lipoarabinomannan containing linear α1,5-Ara polymers and branched α1,2/3-Ara structures which are powerful immunomodulatory lipoglycans (Alderwick *et al.,* 2011; Mishra *et al.,* 2012). If similar structures are present in plant pathogens, they could also constitute important pathogenicity factors. Presumably, they could then be detected by the plant during infection as PAMPs and trigger downstream defense processes (Newman *et al.,* 2013). However, these signals are not targeted into plant cells but are recognized by membrane-bound receptors. In contrast, bacteria like *Pst* DC3000 have developed a sophisticated virulence mechanism involving a type III secretion system, which enables the delivery of a mixture of diverse bacterial effector proteins into the plant cell where they will suppress plant immune responses and promote disease susceptibility (Lindeberg *et al.,* 2012). Glycosylation of secreted proteins by bacteria is rarely reported (Nothaft and Szymanski, 2010).

So, all these carbohydrate-related structures remain speculative interactors for F-box-Nictaba. Therefore, in order to investigate the physiological role of F-box-Nictaba and its interaction with glycans within *A. thaliana* cells, future research should focus on the identification of the interacting partners localized in the nucleus and cytoplasm of plant cells and their characterization in view of possible modification with carbohydrate structures. Certainly, it would be interesting to include both untreated plants, as well as heat-stressed, SA-treated and infected plants to distinguish between normal state and stress-related interactions and to possibly identify targets of non-plant source. Preliminary assays could be performed by a pull-down technique using recombinant F-box-Nictaba and Nictaba domain proteins which are now available (as described in Chapter 2). Results could be further confirmed by more elaborated techniques including tandem affinity purification (Xu *et al.,* 2010) and bimolecular fluorescence complementation for interaction studies *in vivo* (Kerppola,

2008). These experiments would also reveal whether F-box-Nictaba interacts with the components of the SCF machinery, and thus if the protein could function in proteasomal degradation of (glyco)proteins. Additionally, F-box-Nictaba protein variants with a deficient carbohydrate binding activity could be used to validate the relevance of the protein-carbohydrate interactions. Likewise, phenotypic analysis of transgenic *A. thaliana* plants overexpressing such mutant forms of F-box-Nictaba might bring valuable insight in the physiological role of the carbohydrate-binding properties of F-box-Nictaba in Arabidopsis plants.

Furthermore, since F-box-Nictaba encoded by *At2g02360* is just one of the multiple Nictaba homologs containing an N-terminal F-box domain in *A. thaliana*, it would be relevant to extend the research by functional studies of other members of the family. Since the AAs essential for glycan binding activity are conserved in most F-box-Nictaba homologs, it is likely that at least some of them are also functional lectins. Judging from the distinct glycan-binding properties between F-box-Nictaba and Nictaba, other homologs may theoretically exhibit yet another distinct specificity, a specificity comparable to the one of F-box-Nictaba or could present glycan-binding properties similar to those of Nictaba. Consequently, the latter (putative) representatives of the F-box-Nictaba family could still be identifed as functional homologs of the mammalian Fbs proteins playing a role in the ERAD pathway (Yoshida and Tanaka, 2010).

5.2 Stress-inducible expression of *F-box-Nictaba*

As presented in Chapter 3, an extensive expression analysis of *At2g02360,* the gene encoding F-box-Nictaba, has been performed in *A. thaliana* plants which were grown under optimal growth conditions, as well as treated with different plant hormones, biotic and abiotic stresses. Based on the outcome of these experiments, it could be concluded that *F-box-Nictaba* gene expression is generally low and stable during the life cycle of Arabidopsis plants without any obvious tissue specificity. Interestingly, *F-box-Nictaba* expression is significantly up-regulated in plants (1) following treatment with SA, a plant hormone involved in plant defense responses towards pathogen infection, (2) after infection with the virulent hemibiotrophic bacterium *Pst* DC3000, and (3) after heat stress. This stress-inducible expression pattern suggests that F-box-Nictaba has a role in plant stress responses, more particularly in SA-mediated plant-pathogen interactions, presumably via glycan signaling.

SA is an immune signal produced by plants in response to pathogen challenge, and which is essential for providing broad spectrum resistance (Vlot *et al.,* 2009). Upon infection, SA initiates transcriptional reprogramming in order to induce systemic acquired resistance (SAR) crucial for plant survival. Similar to all other plant hormones (Dharmasiri *et al.,* 2013), the SA signaling pathway is tightly regulated via the UPS, although the mode of signal perception somewhat differs and does not include F-box protein(s). Fu *et al.* (2012) have recently identified the receptors for SA, and proposed the mechanism of SA perception and its role in triggering localized programmed cell death (PCD) and establishing SAR (Fig. 5.2). In Arabidopsis, the central role in SAR activation is attributed to the

transcription cofactor named nonexpresser of PR genes 1 (NPR1). Whereas NPR1 is required for basal resistance and for the activation of SAR, it negatively regulates pathogen effector-triggered PCD and suppresses the hypersensitive response (HR) (Rate and Greenberg, 2001; Spoel *et al.,* 2009). It has been demonstrated that NPR1 paralogues, NPR3 and NPR4, are SA receptors which function as the BTB (bric-a-brac–tramtrack–broad complex) adaptors of the Cullin3 Ub ligase and specifically recognize and target NPR1 for proteasomal degradation in a SA-dependent manner (Fu et al., 2012; Fig. 5.2). In the absence of pathogens, NPR1 remains in the cytoplasm as an oligomer. However, upon infection, its disulphide bonds are reduced and transcriptionally active monomers of NPR1 are translocated into the nucleus to act as cofactors for transcription factors inducing defense-related genes (Mou et al. 2003). Nevertheless, NPR1 in the nucleus is further tightly regulated by NPR3 and NPR4 in response to different SA levels. Although both NPR3 and NPR4 are SA receptors and target NPR1 for proteasomal degradation, SA binding differently affects their ability to interact with NPR1 (Fu et al., 2012). Whereas SA promotes the NPR1-NPR3 interaction functioning as a molecular glue, SA binding inhibits the interaction between NPR1 and NPR4. What is more, NPR3 binds SA with lower affinity than NPR4. Consequently, in SA-deficient mutants $CUL3^{NPR4}$ constantly targets NPR1 for degradation leading to enhanced susceptibility (Fig. 5.2A). Thus, plants maintain basal SA levels necessary to block some of the NPR1–NPR4 interactions ensuring the basal resistance (Fig. 5.2B). During pathogen infection however, SA levels increase both locally and systemically and form a concentration gradient from the infection site (Dorey et al., 1997). In infected cell where the SA level is the highest, NPR1 is targeted for proteasomal degradation via $CUL3^{NPR3}$, resulting in the activation of HR and PCD (Fig. 5.2C). In turn, a lower SA level in the neighbouring cells is limitingthe NPR1–NPR3 interaction. Therefore, NPR1 can accumulate, restrict the spread of HR and PCD and establishes SAR (Fig. 5.2D).

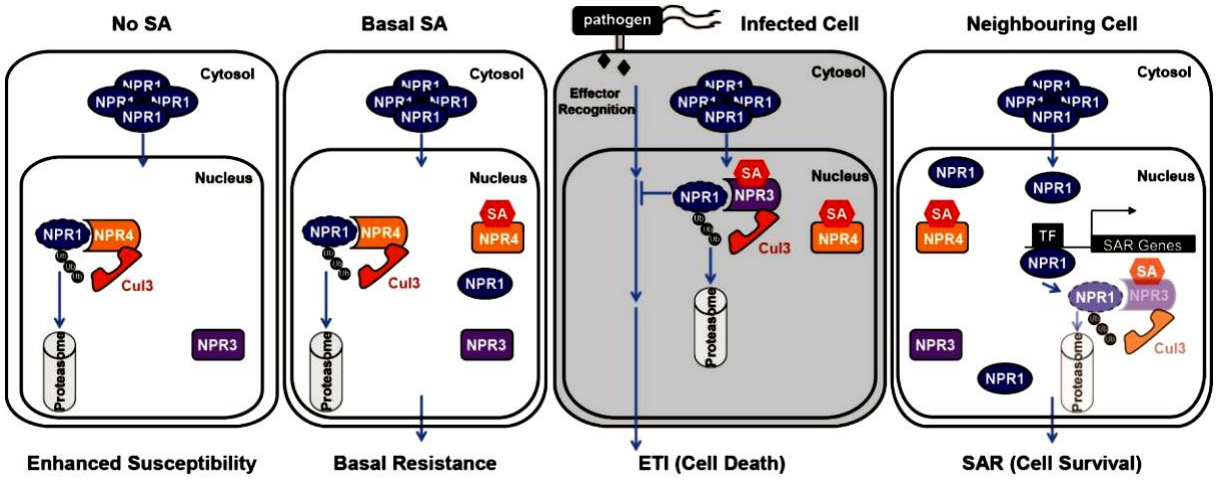


Fig. 5.2 Model of SA perception in determining cell death and survival in response to pathogen challenge (adapted from Fu *et al.,* 2012). A, In SA-deficient plants, $CUL3^{NPR4}$ targets NPR1 for proteasomal degradation causing enhanced susceptibility. B, In WT plants, a basal SA level reduces $CUL3^{NPR4}$-mediated NPR1 degradation by blocking NPR4 and confers basal resistance. C and D, Upon pathogen infection, SA levels increase locally and systemically. The highest level of SA in infected cells promotes $CUL3^{NPR3}$-driven degradation of NPR1 and leads to effector-triggered immunity (ETI) and PCD (C). In the neighbouring cells, lower SA levels limit NPR1-NPR3 interaction and allow NPR1 to accumulate, inhibit PCD and establish SAR (D). Ub, Ubiquitin; TF, transcription factor.

Interestingly, co-expression analyses presented in Chapter 3 revealed that *F-box-Nictaba* is co-expressed with genes encoding NPR1 and NPR4 (*At1g64280* and *At4g19660,* respectively), supporting the putative involvement of F-box-Nictaba in SA-mediated plant-pathogen interactions.

Several other plant lectin-like proteins have been reported to be involved in plant defense against pathogens (Arnaud *et al.,* 2012; Bouwmeester *et al.,* 2011, 2014; Desclos-Theveniau *et al.,* 2012; Singh *et al.,* 2012), and some of them are regulated in a SA-dependent manner (Armijo *et al.,* 2013; Bouwmeester and Govers, 2009; Huang *et al.* 2013). However, most of these lectin-like proteins are reported to be membrane-bound proteins acting as receptors, which could recognize the glycan-containing extracellular PAMPs/DAMPs and effectors (Wirthmueller *et al.,* 2013). In contrast, the anticipated role of F-box-Nictaba in the UPS requires the localization of this protein in the nucleus and/or cytoplasm of plant cells. Indeed, F-box-Nictaba has been previously demonstrated to be a nucleocytoplasmic protein (Lannoo, 2007). Similarly, the lectin-like protein PP2-A1 from Arabidopsis (encoded by *At4g19840*), predicted to be localized in the nucleus (Lannoo, 2007), has been recently demonstrated to have inducible expression in Arabidopsis plants after infection with *Pst* DC3000 and ET, to act as molecular chaperone and exhibited antifungal activity (Lee *et al.,* 2014). Another F-box-Nictaba homolog, named VBF which stands for VIP1-binding F-box protein and encoded by *At1g56250,* showed inducible expression in Arabidopsis plants upon *A. tumefaciens* and *E. coli* infection, and regulated gene expression by directing the VIP1 transcription factor for proteasomal degradation, but its extact role in pathogen infection remains unclear (Wang *et al.,* 2014; Zaltsman *et al.*, 2010). Another F-box-Nictaba homolog AtPP2-B11 (encoded by *At1g80110*) is up-regulated after drought treatment and negatively regulates plant responses to drought stress (Li *et al.,* 2014d). Both *At1g56250* and *At1g80110* homologs have already been experimentally confirmed to encode nuclear or nucleocytoplasmic proteins, although the carbohydrate binding activities of these proteins (VBF and PP2-B11) have not been described yet. Altogether, these data demonstrate that the F-box-Nictaba homolog investigated in this work is not the only stress-inducible representative of this protein family.

Although *F-box-Nictaba* is induced upon SA treatment, it still needs to be confirmed that the expresssion after pathogen infection is indeed SA-dependent. To corroborate this issue, F-box-Nictaba expression during *Pst* DC3000 infection should be also checked in *A. thaliana* plants impaired in the SA pathway. The commonly used Arabidopsis plants to evaluate SA-dependent regulation of physiological processes include *NahG* transgenic lines and *sid2* mutants. *NahG* plants synthesize bacterial salicylate hydroxylase, which leads to substantial reduction in endogenous SA levels (Friedrich *et al.,* 1995). S*id2* plants are mutated in the *ICS1* gene encoding isochorismate synthase required for SA synthesis from chorismate in A. thaliana plants (Wildermuth et al., 2001). Until now, we only performed qRT-PCR experiments on *NahG* plants infected with Pst DC3000 and indeed, no significant up-regulation of F-box-Nictaba could be detected (Results not shown). Yet, these plants are also characterized by the accumulation of catechol upon SA degradation. Apparently, catechol mediates an inappropriate production of hydrogen peroxide and thus may cause effects attributed to SA deficiency (van Wees and Glazebrook, 2003). Additional experiments

with the *sid2* mutant lines impaired in the biosynthesis of SA would certainly allow to draw more definite conclusions.

Furthermore, since in silico promoter analysis indicated that the *At2g02360* promoter sequence contains multiple W-box cis-elements as possible targets for WRKY transcription regulators commonly associated with stress responses (Bakshi and Oelmüller, 2014; Pandey and Somssich, 2009), it is suggested that *F-box-Nictaba* expression is controlled by the SA-inducible WRKY proteins. Also, *F-box-Nictaba* is co-expressed with the NPR1 and NPR4 proteins, which are the key regulators of plant defense against bacterial pathogens activating several WRKY transcription factors (Wang *et al.*, 2006). To verify the hypothesis of WRKY-mediated regulation of *F-box-Nictaba,*,its expression should be analyzed in mutant lines impaired in the synthesis of these transcription factors (e.g. *wrky70*, *wrky18;* Knoth *et al.,* 2007; Wang *et al.,* 2006). Finally, to get better insights in the regulation of *F-box-Nictaba* expression during stress, the *At2g02360* promoter activity could be visualized by a GUS assay on the *pAt2g02360:GUS* plants (Chapter 3) after treatments with SA, pathogen infection and heat stress.

It has been demonstrated that SA signaling, commonly associated with pathogen infection, is also playing an important role in thermotolerance in plants (Clarke *et al.,* 2004, 2009; Larkindale *et al.,* 2005; Zhang and Wang, 2011). Even though the exact mechanism behind SA involvement in plant responses to heat stress is still unknown, their signaling crosstalk is evident. For example, pre-treatment with SA potentiates the accumulation of heat shock proteins after temperature stress (Cronjé *et al.,* 2004; Snyman and Cronjé, 2008). NPR1, the main regulator of the SAR (mediated by SA), is also largely involved in plant thermotolerance (Clarke *et al.,* 2004; Zhang and Wang, 2011). For instance, WRKY39 has been identified as a transcription factor responding to both heat stress as well as SA application and pathogen infection (Dong *et al.,* 2003; Li *et al.,* 2010). As such, WRKY39 could be a good candidate to be analyzed as possible regulator of *F-box-Nictaba* gene expression by using e.g. the *wrky39* mutant *A. thaliana* plants.

The transgenic Arabidopsis plants overexpressing F-box-Nictaba only showed reduced leaf damage after infection with the virulent bacterium *Pst* DC3000. Nonetheless, the phenotypic analyses described in Chapter 3 should be extended by more detailed analyses including the assessment of bacterial growth, cell death and reactive oxygen species (ROS) production to get a better insight into the plant defense responses underlying the phenotype. Since KO plants did not exhibit any obvious differential behaviour at neither phenotypic or molecular level after stress treatments most probably due to protein redundancy, generation of double mutants for pairs of F-box-Nictaba homologs could be considered. Alternatively, in view of the high sequence similarity betweeen different F-box-Nictaba homologs from Arabidopsis, several homologs could be targeted for silencing using appropriately designed RNAi constructs.

As demonstrated in Chapter 3, infection with the necrotrophic fungus *B. cinerea* lead to slight down-regulation of *F-box-Nictaba* gene expression. It has been reported that the fungus can manipulate the plant defense machinery for its own benefit (El Oirdi *et al.,* 2011; La Camera *et al.,* 2011).

Therefore it would be interesting to check the performance of transgenic plants subjected to infection with *B. cinerea*. On the other hand, expression analysis of *F-box-Nictaba* in Arabidopsis plants after infection with an avirulent *Pseudomonas* strain could clarify whether F-box-Nictaba might also play a role in the gene-for-gene plant resistance (Martin *et al.,* 2003). Moreover, even though the reduction in overexpression of *F-box-Nictaba* gene in the transgenic lines subjected to heat stress highlights the relevance of F-box-Nictaba in plant responses to elevated temperature, it is rather puzzling why no similar effect was present after pathogen infection. The reason for such strict and directed negative regulation as well as the underlying molecular mechanism still remain open questions to be addressed in the future.

Finally, in order to investigate F-box-Nictaba involvement in plant responses to both biotic and abiotic stresses, physiological experiments with a combination of stress applications using pretreatments with SA/heat stress could be performed on the transgenic plants overexpressing *F-box-Nictaba* gene and plants impaired in its expression. Taking into account that in their natural environment plants are constantly and simultaneously exposed to multiple stress factors of both abiotic and biotic origin, identification of the molecular points of convergence is of crucial importance for the development of broad-spectrum stress-tolerant crop plants. Indeed, this approach has become recently the focus of plant research (Abuqamar *et al.,* 2009; Atkinson and Urwin, 2012; Kissoudis *et al.,* 2014; Rasmussen *et al.,* 2013; Qiao *et al.,* 2013).

Another issue is whether the physiological role of F-box-Nictaba in plant stress responses relies on the UPS signaling. Indeed, F-box-Nictaba has already been confirmed by yeast two hybrid screening to interact with four different ASK proteins, namely ASK1, ASK2, ASK11 and ASK12 (AIMC, 2011; Takahashi *et al.,* 2004). ASKs are adaptor proteins enabling interaction of F-box proteins with the rest of the SCF-type Ub ligase. Thus, it seems that F-box-Nictaba could form the $SCF^{F\text{-box-Nictaba}}$ complex and target specifically recognized (glyco)proteins for proteasomal degradation. As shown by the results of plant treatment with MG132, proteasome inhibition did not affect *F-box-Nictaba* gene expression. Typically, proteasome inhibition is performed to check whether a specific protein undergoes proteasomal degradation and not to investigate gene expression. Nonetheless, theoretically we could expect that *F-box-Nictaba* could be induced, since the target for F-box-Nictaba would not be efficiently eliminated from the cells. However, it does not have to be the case. F-box-Nictaba could be tightly regulated posttranscriptionally or posttranslationally rather than at transcriptional level. Alternatively, it could be that F-box-Nictaba does not target (glyco)proteins for proteasomal degradation. Although the best characterized role of Ub is related to the selective protein degradation via the 26S proteasome, ubiquitination constitutes a much more elaborated regulatory mechanism which, apart from targeting proteins for proteasomal degradation, controls a variety of cellular processes in plants including protein (in)activation, localization, modulation of protein-protein interactions, DNA repair and gene transcription (Walsh and Sadanandom, 2014). These diverse effects are related to different types of substrate ubiquitination: proteins can be mono-, multimono- or as well as polyubiquitinated. Furthermore, each Ub contains seven lysines as potential points of ubiquitination by another Ub: K6, K11, K27, K29, K33, K48, and K63 (Saracco et al., 2009;

Walsh and Sadanandom, 2014). Hence, if F-box-Nictaba indeed functions in SCF compexes, it might equally well label its substrates with non-K48-linked Ub chains and affect these proteins in a different way than by tagging for degradation. Alternatively, F-box-Nictaba has a UPS-independent role as it has been already demonstrated for other F-box proteins (Galan *et al.,* 2001; Hermand *et al.,* 2003; Kitagawa *et al.*, 1999; Smaldone *et al.,* 2004; Nelson *et al.,* 2007; Yoshida *et al.,* 2007). Certainly, future studies should investigate the putative function of F-box-Nictaba in the UPS. Interaction studies including tandem affinity purification (Xu *et al.,* 2010) and bimolecular fluorescence complementation (Kerppola, 2008) would evaluate if indeed the protein can interact with different ASK proteins *in vivo.* Furthermore, interaction experiments could be performed by a pull-down technique using the available recombinant F-box-Nictaba and Nictaba domain proteins, to identify possible substrates of F-box-Nictaba and determine how their stability in plants is affected by proteasome inhibition. Also it should be checked what is the effect of F-box-Nictaba knockout and overexpression in plants on the prevalence and ubiquitination state of the target proteins, both in normal growth condiitions as well as after stress application. Finally, different components of the $SCF^{F\text{-box-Nictaba}}$ complex as well as the putative targets could be recombinantly expressed and used in ubiquitination assays to reconstitute and confirm the presumed mechanism *in vitro* (Petroski and Deshaies, 2005b).

5.3 F-box-Nictaba occurence in trichomes

GUS histochemical staining experiments performed on transgenic plants (Chapter 3) allowed to visualize a preferential *F-box-Nictaba* promoter activity localized in the leaf trichomes. The hypothesis of pronounced expression of F-box-Nictaba in trichomes has been further confirmed by qRT-PCR and immunodetection analyses on isolated trichomes (Chapter 4). These results were also supported by the identification of *cis*-regulatory elements responsible for trichome-specific gene regulation in the *F-box-Nictaba* promoter (Ni *et al.,* 2008; Shangguan *et al.,* 2008). Even though substantial dissimilarities in the prevalence level have been reported between two different *A. thaliana* ecotypes tested, the trichome-specific expression of F-box-Nictaba in Ler-0 background was evident. In fact, apparently Col-0 and Ler-0 ecotypes present generally differential trichome-related phenotypes including trichome density and spacing (Larkin *et al.,* 1996).

The strikingly high up-regulation of the *F-box-Nictaba* gene in the trichomes of Ler-0 plants lead us to consider a possible trichome-specific role distinct from its role in other plants cells. Multiple genes with trichome-specific expression, e.g. *GL1, GL3, TRY, CPC, GL2,* are encoding key regulators of trichome development and their mutations lead to diverse abnormal trichome-related phenotypes (Kirik *et al.,* 2005; Maes *et al.,* 2008; Rerie *et al.,* 1994*;* Wester *et al.,* 2009). However, transgenic F-box-Nictaba-deficient plants and plants overexpressing F-box-Nictaba do contain trichomes, thus the F-box-Nictaba trichome-specific expression does not seem pivotal for trichome development. Nonetheless, effects of F-box-Nictaba overexpression and knockout in trichomes should be still

investigated at the molecular level and it should be checked in more detail if trichome density and structure are not affected in these transgenic plants.

Based on the emerging involvement of the Arabidopsis non-glandular trichomes in stress signaling and as a source of chemical-based defense against invaders (Bruner, 2009; Calo *et al.,* 2006; Frerigmann *et al.,* 2012; Wienkoop *et al.*, 2004), it is tempting to speculate that F-box-Nictaba expression in trichomes might be implicated in plant stress responses, possibly via glycan signaling. Indeed, Marks *et al.* (2008) showed that arabinose-containing carbohydrates are abundantly present in the cell walls of Arabidopsis trichomes. In contrast, although Lewis A glycan structures recognized by F-box Nictaba are reported at very low levels in Arabidopsis (Fitchette *et al.,* 1999; Léonard *et al.*, 2002; Melo *et al.,* 1997; Strasser *et al.,* 2007), yet no information is available regarding their occurence in trichomes. In Chapter 4 we were able to demonstrate that the enzymes indispensable for Lewis A synthesis in *A. thaliana* were significantly up-regulated in the trichomes of WT *A. thaliana* with Ler-0 background. Of course, this does not prove the efficient synthesis of Lewis A motifs in trichomes and does not indicate that they would be accesible to F-box-Nictaba. Preliminary immunodetection studies using the JIM84 antibody specific for Lewis A motifs (Fitchette *et al.,* 1999) performed on protein extracts from isolated trichomes unfortunately did not bring satisfactory results. Further experiments are required to confirm the presence and, more importantly, cellular localization of Lewis A structures in trichomes. Similarly, the subcellular localization of F-box-Nictaba in the trichomes should be investigated. Even though transgenic *A. thaliana* plants overexpressing an EGFP-fusion construct of F-box-Nictaba have been generated, no recombinant protein could be detected (Results not shown). As an alternative approach, immunolocalization of the native protein could be performed in the isolated trichomes, both in untreated plants and after SA/heat-stress application (Zhang and Oppenheimer, 2004). It would also be worthwhile to analyze the expression of the genes encoding different glycosyltransferases, genes involved in plant defense against pathogens and those related to heat stress in the trichomes isolated from transgenic *A. thaliana* plants impaired in F-box-Nictaba expression and those overexpressing the protein.

Furthermore, the question remains if F-box-Nictaba would still function in trichomes as a component of the SCF complex mediating selective glycoprotein degradation via the proteasome, or whether it might play a UPS-independent role, as it has been demonstrated for mammalian F-box proteins with a highly tissue-specific expression (Jonkers and Rep, 2009; Nelson *et al.,* 2007; Yoshida *et al.,* 2007; Zhao *et al.,* 2013). Clearly, the UPS in trichomes is a fully functional and very active machinery: it post-translationally controls the key regulators of trichome development and it is implicated in the endoreduplication process (Pattanaik *et al.,* 2014). Since at the moment identification of the putative F-box-Nictaba binding partners is certainly the general bottleneck of the research, interaction studies suggested in previous section should definitely also be directed towards unraveling the trichome-specific aspect of F-box-Nictaba molecular interactions.

Lastly, trichomes and trichome-specific promoters are recently gaining attention as potent targets for bioengineering of transgenic plants enhanced in pest and pathogen resistance (Tissier, 2012b).

Therefore, studies of a carbohydrate-binding F-box protein involved in glycan-mediated signaling in trichomes could provide novel ideas for the potential exploitation of these structures in plant protection from adverse environmental conditions.

5.4 Post-translational regulation of F-box-Nictaba

Throughout the experimental research presented in this work an unexpected molecular size of F-box-Nictaba was a recurring issue. Although, based on the AA sequence, the predicted MW of F-box-Nictaba should be 31.3 kDa, we showed that F-box-Nictaba recombinantly expressed as a secreted protein in *P. pastoris* was detectable in some of the cultures as two distinct bands with a much higher MW (approximately 55 kDa) (Chapter 2). Transgenic overexpression *A. thaliana* plants also synthesized F-box-Nictaba as a protein of high MW. However in this case, only a single band estimated at approximately 70 kDa was detected (Chapter 3). Finally, native F-box-Nictaba detected in protein extracts from trichomes presented a double-band pattern with calculated MW of 61.4 kDa and 68.1 kDa (Chapter 4). The repeated occurrence of F-box-Nictaba as a much larger protein than anticipated in different biological systems and in independent experiments, often detectable as two clearly separate protein bands on Western blot, is very intriguing and could point towards a possible protein modification. *In silico* analyses revealed that the F-box-Nictaba sequence contains putative regions for post-translational modifications including one putative *N*-glycosylation site (Schwarz *et al.,* 2011; Strasser, 2014), six *O*-GlcNAcylation sites (Hart and Akimoto, 2009), three *N*-myristoylation sites (Boisson *et al.,* 2003; Podell and Gribskov, 2004) and two SUMOylation sites (Park *et al.,* 2011) (Fig. 4.5). Recombinant F-box-Nictaba protein produced in *P. pastoris,* characterized by only a slightly higher MW, was indeed demonstrated to be *N*-glycosylated. Yet it is difficult to associate a shift by > 30 kDa to *N*-glycosylation, especially in view of the fact that only one putative *N*-glycosylation site is present in the sequence. What is more, F-box-Nictaba was shown to localize to the nucleus and cytoplasm of plant cells and is not assumed to be secreted in *A. thaliana* (Lannoo, 2007). Another reason could be *O*-GlcNAcylation, a modification found on some plant nuclear proteins (Heese Peck *et al.,* 1995; Heese Peck and Raikhel, 1998), but here again even the modification of all six available sites should not result in such a significant shift in MW. Possible glycosylation of the F-box-Nictaba expressed in plant could be verified by comparing its size before and after deglycosylation. In contrast, SUMOylation seems much more probable, since one SUMO molecule corresponds to 12 kDa and SUMOylation may involve protein modification with multiple SUMO units (Park *et al.,* 2011). Importantly, SUMOylation affects plant responses to environmental stresses including heat stress and defense reactions to pathogen infection. Protein modification with SUMO regulates its subcellualar localization, modulates protein-protein interactions and influences protein stability by antagonizing ubiquitination (Geiss-Friedlander and Melchior, 2007; Gill, 2004). Experiments on the SUMOylation of F-box-Nictaba are currently being performed in the lab to investigate this hypothesis.

Nevertheless, the observed MW of F-box-Nictaba roughly corresponds to double of what we would expect. Thus, it could be also speculated that, similarly to its prototype protein from tobacco (Nictaba, Chen *et al.,* 2002), F-box-Nictaba forms dimers and reducing conditions of SDS-PAGE were not harsh enough to break it. There are eight Cys residues within the AA sequence of F-box-Nictaba which could theoretically participate in disulphide bond formation (Fig. 4.5). Provided that F-box-Nictaba dimerizes, the estimated MW of the dimer would be approximately 62.5 kDa. Although stable disulphide bonds are typically formed in the ER in secreted or membrane proteins and are not commonly found in the cytosol where the conditions are reducing, it has been demonstrated that stress conditions like pathogen infection and heat shock cause cellular redox changes (Baxter *et al.,* 2013; Mou *et al.,* 2003). In fact, generation of ROS is characteristic for plant responses to both biotic and abiotic stresses and is proposed as a key process in the crosstalk (Baxter *et al.,* 2013). SA has been found to induce changes in the plant antioxidant system and to protect against heat-induced oxidative damage by acting as scavenger of ROS (Clarke *et al.,* 2004; Dat *et al.,*1998; Larkindale and Knight, 2002). An excellent example of protein regulated through the redox changes in the cytoplasm is the NPR1 protein. It is retained in the cytoplasm as an oligomer through redox-sensitive intermolecular disulphide bonds (Peleg-Grossmanet *et al.,* 2010). However, after pathogen challenge, the disulphide bridges are reduced, and NPR1 monomers are released into the nucleus to function as transcription cofactors (Mou *et al.,* 2003). Another cytoplasmic protein from Arabidopsis, thioredoxin AtTrx-h3, forms various protein structures ranging from low and oligomeric protein species to high MW complexes, depending on the heat shock and on the cellular redox status (Park *et al.,* 2009). Interestingly, these various forms of the protein are associated with different activities. Under normal conditions monomeric/dimeric AtTrx-h3 functions as disulphide reductase, whereas after oxidative and heat stress the predominant multimeric form of the protein plays a role as molecular chaperone. In fact, also several F-box proteins funtioning in the UPS in yeast and mammals do dimerize. Apparently, dimerization of F-box proteins might influence substrate ubiquitination (Hao *et al.,* 2007; Kominami *et al.,* 1998; Li and Hao, 2010; Suzuki *et al.,* 2000; Tang *et al.,* 2007; Welcker and Clurman, 2007). Also, some F-box proteins form heterodimers with Skp1 proteins and act independently of SCF-mediated degradation process as transcription cofactors, cell cycle regulators, in vesicle trafficking (Galan *et al.,* 2001; Hermand *et al.,* 2003; Kitagawa *et al.*, 1999; Smaldone *et al.,* 2004), or, like the mammalian Fbs proteins, play a role as chaperones to prevent aggregate formation (Nelson *et al.,* 2007; Yoshida *et al.,* 2007).

Disulphide bond formation can be actually predicted *in silico*, e.g. using DiANNA software for Cys state and disulphide bond partner prediction (http://clavius.bc.edu/~clotelab/DiANNA/). The software relies on the presence, location and oxidation state prediction of Cys residues. It indicates that there are four possible disulphide bonds within the F-box-Nictaba polypeptide. Input of the doubled F-box-Nictaba AA sequence returns four disulphide bonds that might occur with high probability between the two polypeptides. In order to verify the hypothesis of F-box-Nictaba dimerization, size-exclusion chromatography preserving macromolecular interactions could be performed combined with native PAGE.

Clearly, the possibility of F-box-Nictaba (homo/hetero)dimerization and its post-translational modification(s) *in vivo* need to be further studied in more detail, especially in view of the putative implication of these events for the functionality of F-box-Nictaba in the UPS and its physiological role in plants.

5.5 Conclusive remarks

To conclude, the purpose of the research presented in this work was to elucidate the physiological role of a putative carbohydrate-binding F-box protein from *A. thaliana,* F-box-Nictaba. The first objective was to investigate the anticipated lectin activity of the protein. It has been demonstrated that F-box-Nictaba is a functional lectin and can bind, through its C-terminal Nictaba domain, to plant-type glycans including Lewis A motifs and arabino-oligosaccharides. Characterization of the F-box-Nictaba expression profile in *A. thaliana* plants was the second goal of the research which was accomplished. It was shown that F-box-Nictaba is continuously expressed throughout the lifecycle of plants at a relatively low level. However, its expression is induced after stress application including SA, pathogen attack and heat stress. It was also demonstrated that F-box-Nictaba is preferentially expressed at high levels in the trichomes. The third aim of the project was to show the relevance of F-box-Nictaba for plant physiology. Although the underlying mechanism is unknown, it was shown that the lectin is involved in plant defense responses against pathogen infection and heat stress.
Altogether, this study provided evidence for the occurrence of a sugar-binding F-box protein in plants and for its role in plant stress physiology. Whether its function in plants is UPS-dependent and relies on the carbohydrate-binding activity, remain open questions until now.

Addendum

SUPPLEMENTARY FIGURES

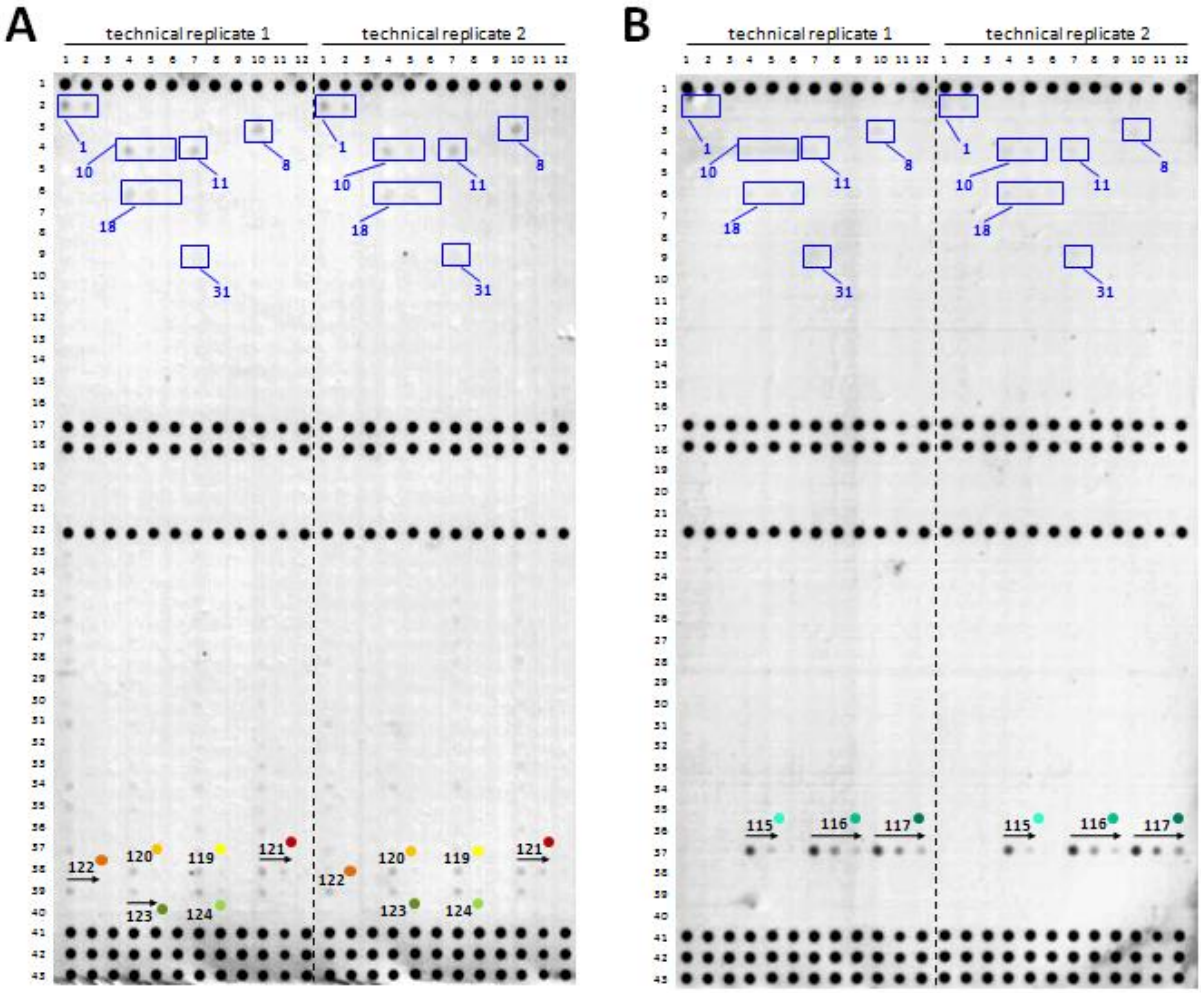


Fig. A2. 1 Grayscale images of the scanned plant glycan microarray probed with a recombinant F-box-Nictaba from Arabidopsis (A) and recombinant Nictaba from tobacco (B) tested at 10 µg/ml and immunodetected as described in Materials and methods. Glycan structures of highest reactivity for each of the analyzed proteins are marked with their glycan ID and colors correspond to the bars in Fig. A2.3 and A2.4. Arrows indicate decreasing concentration of the detected glycan sample. Blue boxes mark glycans for which the reaction appears to be aspecific.

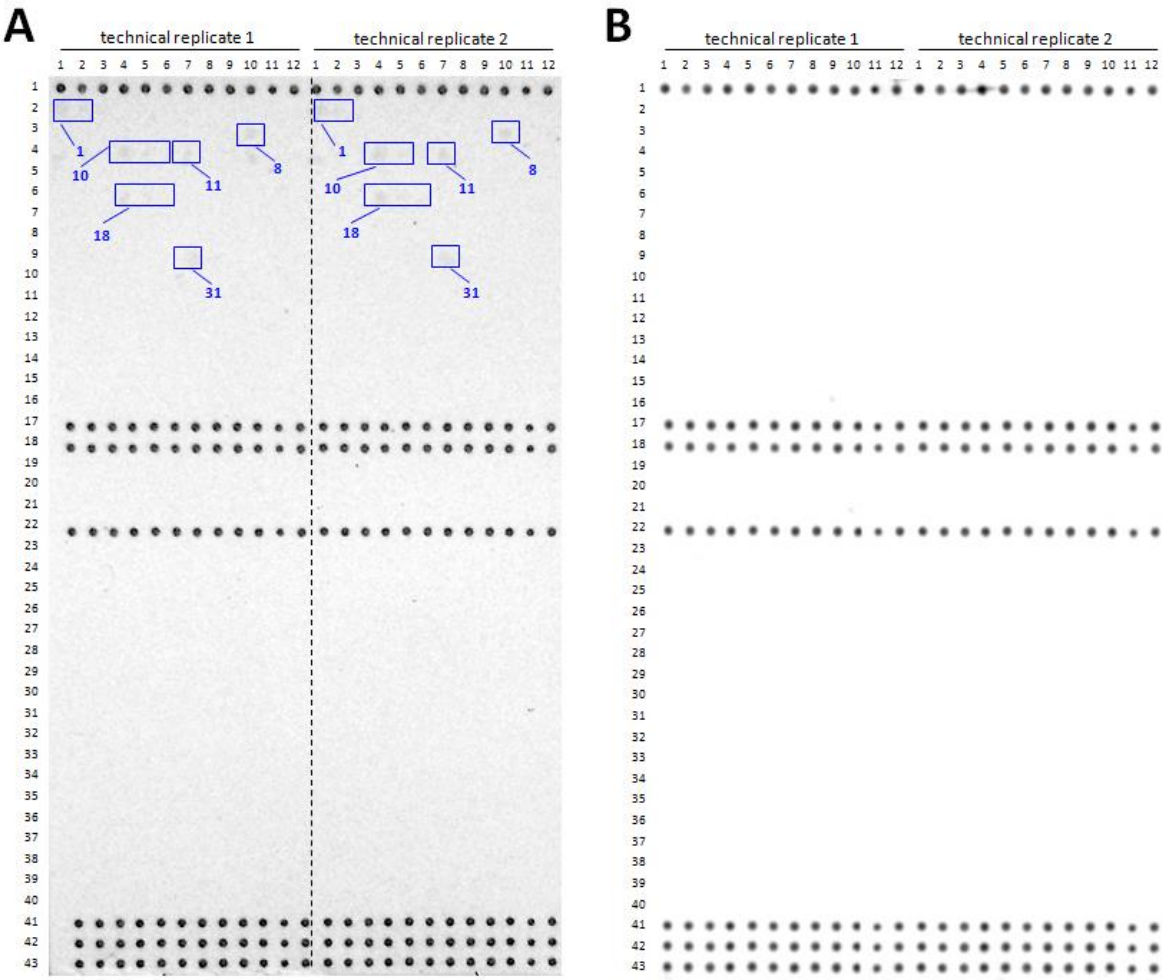


Fig. A2. 2 Grayscale images of the negative controls for the plant glycan microarray screening including the scanned non-probed microarray before the experiment (A) and the scanned microarray probed with antibodies used to detect His_6-tagged proteins (no incubation with a lectin) and immunodetected (B). Blue boxes mark glycans already visible on the array.

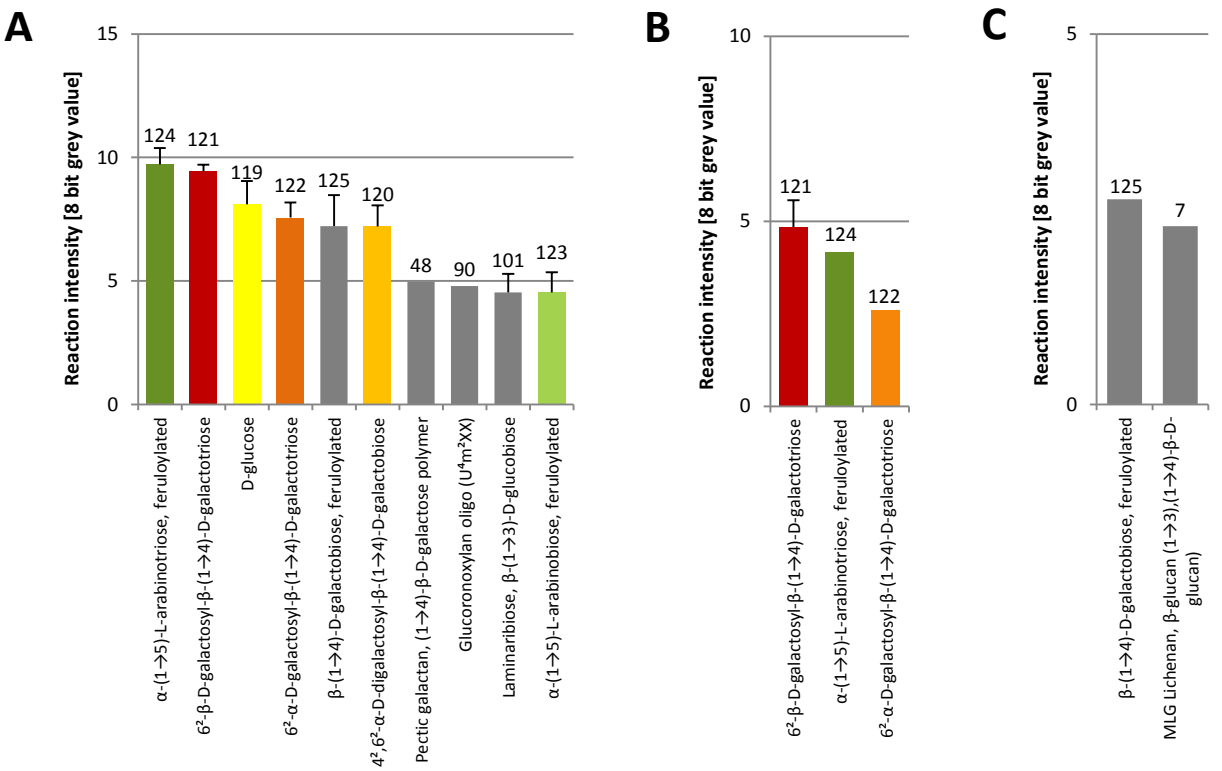


Fig. A2. 3 Result of the plant glycan array screening with F-box-Nictaba from *A. thaliana* tested at 10 µg/ml. Results are shown for glycans printed at (A) highest (2 mg/ml), (B) intermediate (0.4 mg/ml) and (C) lowest (0.08 mg/ml) concentration. Reaction intensities are presented for top 10 glycan structures for each glycan dilution with highest reactivity on the array. For glycans detectable in two technical replicates, bars represent their mean value with SD. Colors are representing glycans as in Fig A2.1A.

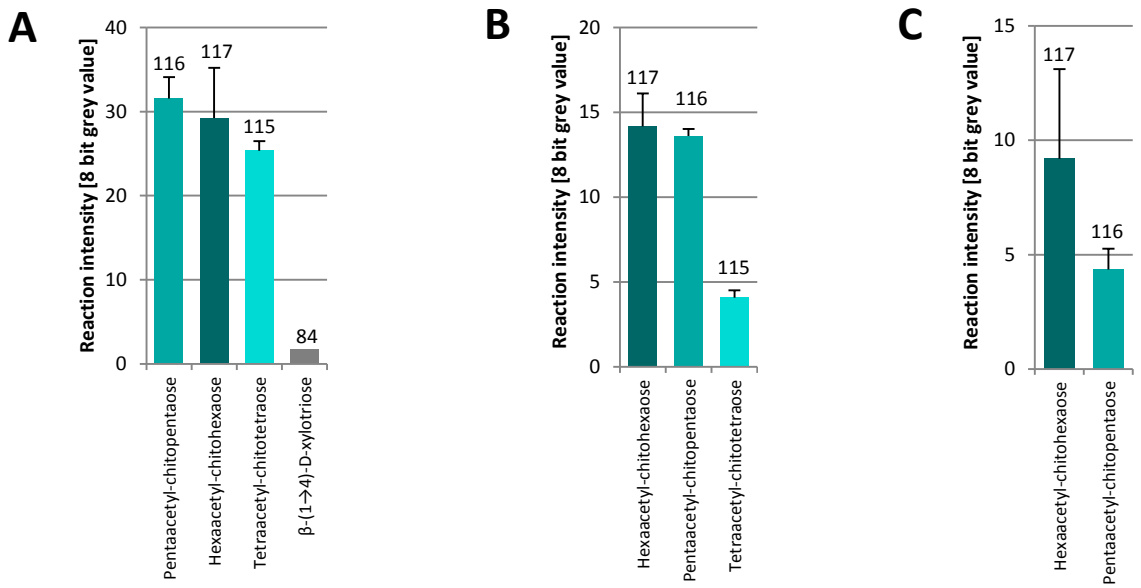


Fig. A2. 4 Result of the plant glycan array screening with Nictaba from tobacco tested at 10 µg/ml. Results are shown for glycans printed at (A) highest (2 mg/ml), (B) intermediate (0.4 mg/ml) and (C) lowest (0.08 mg/ml) concentration. Reaction intensities are presented for top 10 glycan structures for each glycan dilution with highest reactivity on the array. For glycans detectable in two technical replicates, bars represent their mean value with SD. Colors are representing glycans as in Fig A2.1B.

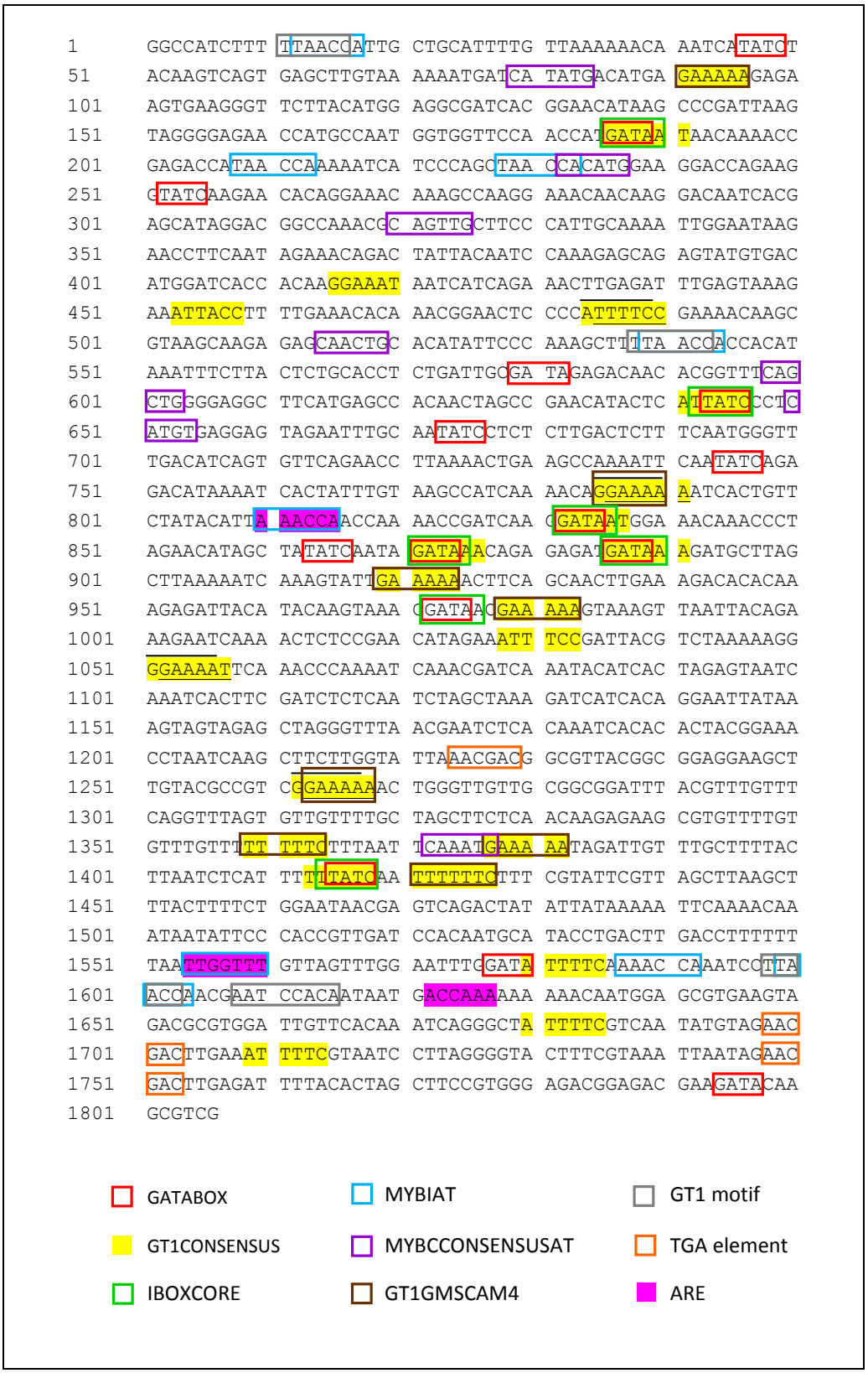


Fig. A3. 1 *At2g02360* promoter sequence, with major putative *cis*-acting regulatory elements (results according to Place database) highlighted.

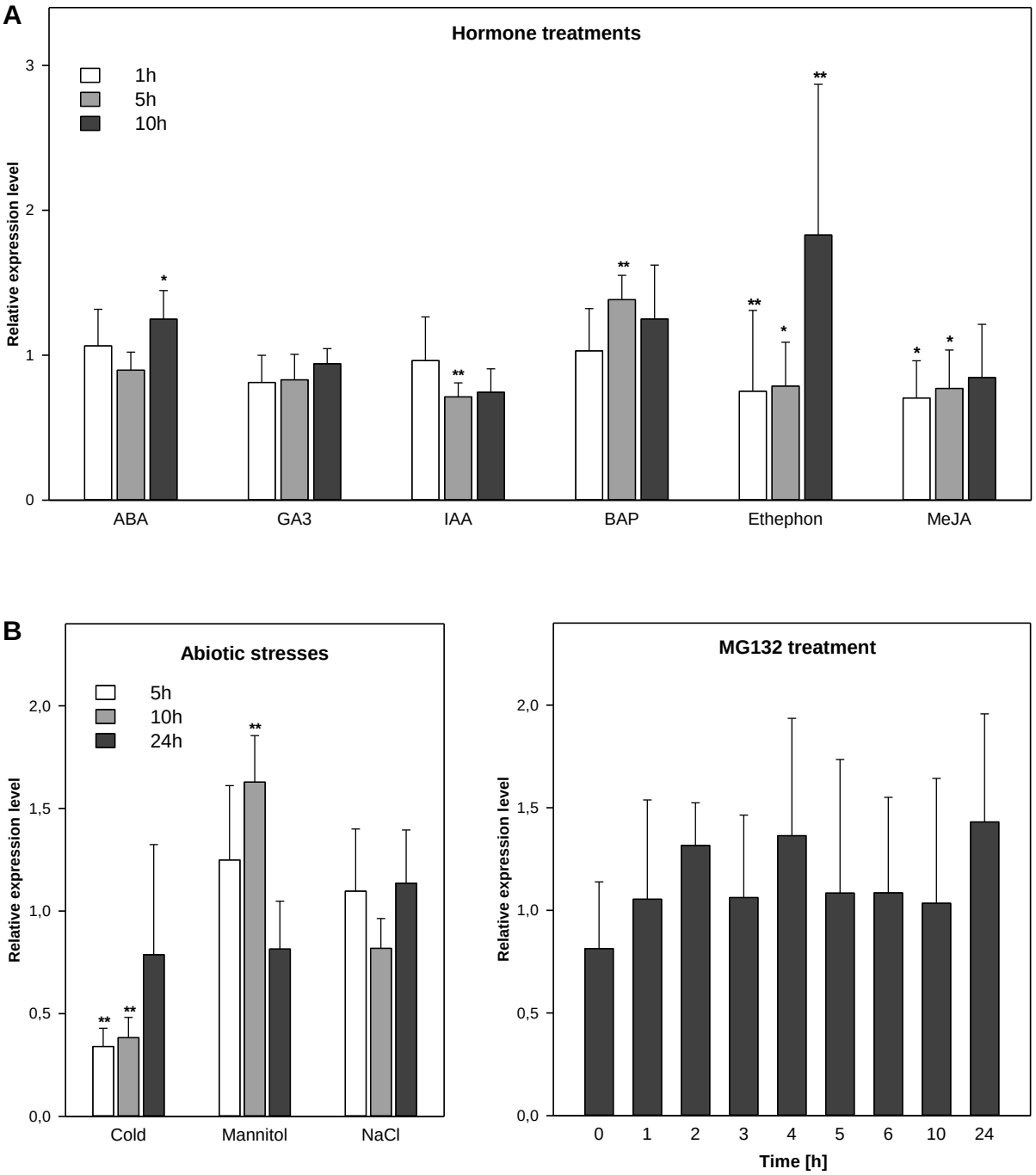


Fig. A3. 2 Relative transcript levels of *At2g02360* in 16-day-old WT *A. thaliana* Col-0 seedlings determined by qRT-PCR analyses of two independent biological experiments. *n*=2; error bars ± SE. Asterisks indicate statistically significant differential expression compared to control samples (*p<0.05; **p<0.01). A, *At2g02360* expression levels after treatment with 100 µM plant hormones. B, *At2g02360* expression levels after treatment with cold stress (4°C), 100 mM mannitol and 150 mM NaCl. C, *At2g02360* expression levels in seedlings treated with 50 µM MG132.

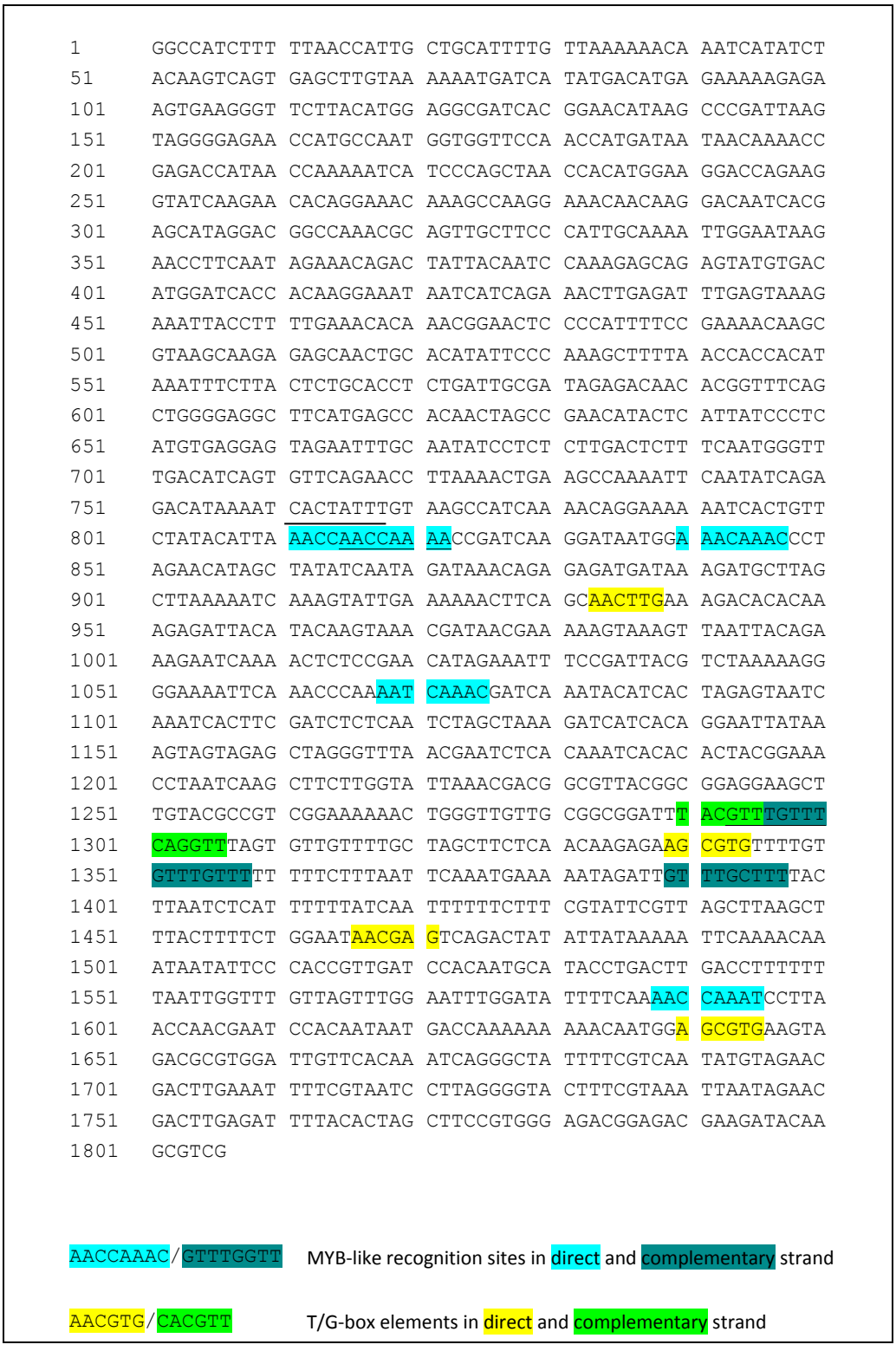


Fig. A3. 3 *At2g02360* promoter sequence, with highlighted putative *cis*-acting regulatory elements possibly involved in promoter activity in trichomes. Sequence analysis was performed with one mismatch allowed.

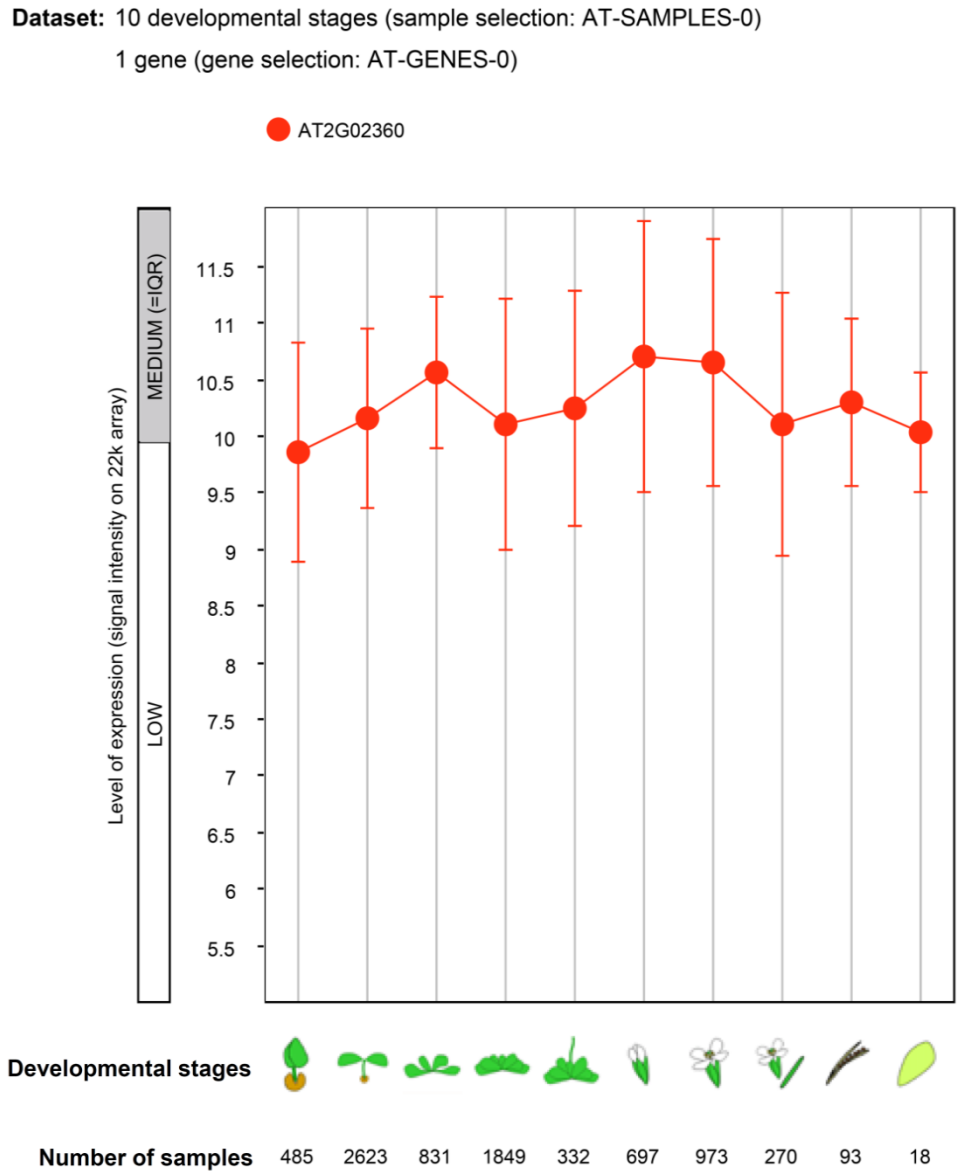


Fig. A3. 4 Level of *At2g02360* expression across the lifecycle of WT *A. thaliana* Col-0 plants generated using the Genevestigator search tool.

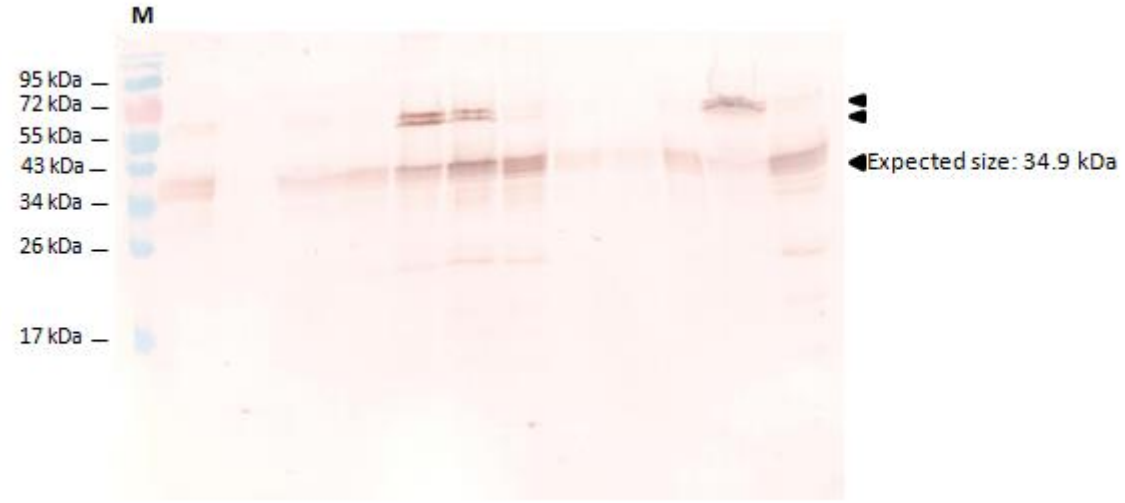


Fig. A4. 1 Western blot of crude protein extracts from media of *P. pastoris* cultures transformed with constructs for recombinant F-box-Nictaba protein expression. Protein extracts were prepared from 1 ml of medium originating from different cultures transformed with constructs for recombinant expression of F-box-Nictaba grown at 22°C and treated with 1% methanol. Immunodetection was performed using an anti-His antibody. M: protein marker.

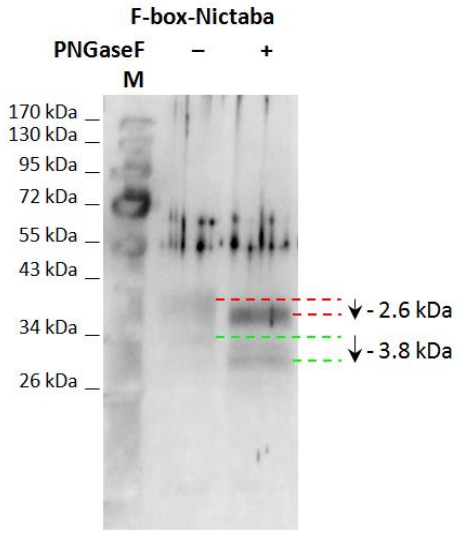


Fig. A4. 2 Western blot on purified recombinant F-box-Nictaba protein before (-) and after (+) PNGaseF treatment. Immunodetection was performed using a specific anti-F-box-Nictaba antibody. M: protein marker.

SUPPLEMENTARY TABLES

Table A2. 1 Overview of primers used in molecular cloning of constructs for recombinant protein expression in *P. pastoris*.

Target gene/sequence	**Forward primer (5'-3')**	**Reverse primer (5'-3')[a]**
Full-length *F-box-Nictaba*	evd553 GGACACGTGGGGGAGAAAACGCAGAGTTAAATCGG	evd554 GCTTCCGCGGCGAGGATTTTAGCAGGTCGGATTTC
Sequence encoding Nictaba domain of F-box-Nictaba	evd360 GGCGGAGAATTCAGCGTATGGTTAGAGAAAGCGAGTGGG	evd359 CCCGCTTGCGGCCGCGAGGATTTTAGCAGGTCGGATTTC
AOX1	evd21 GACTGGTTCCAATTGACAAGC	evd22 GCAAATGGCATTCTGACATCC

Table A2. 2 Layout of the plant glycan microarray used in this study. Glycans are designated with numerical IDs as presented in Table A2.3.

	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
1	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink
2	1	1	1	2	2	2	3	3	3	4	4	4	1	1	1	2	2	2	3	3	3	4	4	4
3	5	5	5	6	6	6	7	7	7	8	8	8	5	5	5	6	6	6	7	7	7	8	8	8
4	9	9	9	10	10	10	11	11	11	12	12	12	9	9	9	10	10	10	11	11	11	12	12	12
5	13	13	13	14	14	14	15	15	15	16	16	16	13	13	13	14	14	14	15	15	15	16	16	16
6	17	17	17	18	18	18	19	19	19	20	20	20	17	17	17	18	18	18	19	19	19	20	20	20
7	21	21	21	22	22	22	23	23	23	24	24	24	21	21	21	22	22	22	23	23	23	24	24	24
8	25	25	25	26	26	26	27	27	27	28	28	28	25	25	25	26	26	26	27	27	27	28	28	28
9	29	29	29	30	30	30	31	31	31	32	32	32	29	29	29	30	30	30	31	31	31	32	32	32
10	33	33	33	34	34	34	35	35	35	36	36	36	33	33	33	34	34	34	35	35	35	36	36	36
11	37	37	37	38	38	38	39	39	39	40	40	40	37	37	37	38	38	38	39	39	39	40	40	40
12	41	41	41	42	42	42	43	43	43	44	44	44	41	41	41	42	42	42	43	43	43	44	44	44
13	45	45	45	46	46	46	47	47	47	48	48	48	45	45	45	46	46	46	47	47	47	48	48	48
14	49	49	49	50	50	50	51	51	51	52	52	52	49	49	49	50	50	50	51	51	51	52	52	52
15	53	53	53	54	54	54	55	55	55	56	56	56	53	53	53	54	54	54	55	55	55	56	56	56
16	57	57	57	B	B	B	B	B	B	B	B	B	57	57	57	B	B	B	B	B	B	B	B	B
17	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink
18	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink
19	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
20	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
21	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
22	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink
23	58	58	58	59	59	59	60	60	60	61	61	61	58	58	58	59	59	59	60	60	60	61	61	61
24	62	62	62	63	63	63	64	64	64	65	65	65	62	62	62	63	63	63	64	64	64	65	65	65
25	66	66	66	67	67	67	68	68	68	69	69	69	66	66	66	67	67	67	68	68	68	69	69	69
26	70	70	70	71	71	71	72	72	72	73	73	73	70	70	70	71	71	71	72	72	72	73	73	73
27	74	74	74	75	75	75	76	76	76	77	77	77	74	74	74	75	75	75	76	76	76	77	77	77
28	78	78	78	79	79	79	80	80	80	81	81	81	78	78	78	79	79	79	80	80	80	81	81	81
29	82	82	82	83	83	83	84	84	84	85	85	85	82	82	82	83	83	83	84	84	84	85	85	85
30	86	86	86	87	87	87	88	88	88	89	89	89	86	86	86	87	87	87	88	88	88	89	89	89
31	90	90	90	91	91	91	92	92	92	93	93	93	90	90	90	91	91	91	92	92	92	93	93	93
32	94	94	94	95	95	95	96	96	96	97	97	97	94	94	94	95	95	95	96	96	96	97	97	97
33	98	98	98	99	99	99	100	100	100	101	101	101	98	98	98	99	99	99	100	100	100	101	101	101
34	102	102	102	103	103	103	104	104	104	105	105	105	102	102	102	103	103	103	104	104	104	105	105	105
35	106	106	106	107	107	107	108	108	108	109	109	109	106	106	106	107	107	107	108	108	108	109	109	109
36	110	110	110	111	111	111	112	112	112	113	113	113	110	110	110	111	111	111	112	112	112	113	113	113
37	114	114	114	115	115	115	116	116	116	117	117	117	114	114	114	115	115	115	116	116	116	117	117	117
38	118	118	118	120	120	120	119	119	119	121	121	121	118	118	118	120	120	120	119	119	119	121	121	121
39	122	122	122	123	123	123	124	124	124	125	125	127	122	122	122	123	123	123	124	124	124	125	125	127
40	126	126	126	127	127	125	B	B	B	B	B	B	126	126	126	127	127	125	B	B	B	B	B	B
41	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink
42	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink
43	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink

Concentrations of printed glycans	**[mg/ml]**		
Samples 1 - 57: Polysaccharides	1	0,2	0,04
Samples 58 - 127: Oligosaccharides	2	0,4	0,08

NOTE: Some of the gum and β-glucan samples (IDs 8-11 and 22–28) have been printed at different concentrations (as indicated above) than the remaining polysaccharides due to issues with too high viscosity which may create satellites (smaller and unprecise dots). Furthermore, there is a mistake in the 3rd dilution of samples 125/127 – these have been swapped around, as indicated in the layout.

Table A2. 3 List of glycans printed on the plant glycan microarray with corresponding numerical IDs.

ID	Glycan name
1	Mannan (ivory nut)
2	Galactomannan (carob)
3	Glucomannan (konjac)
4	Xylan (birch)
5	Arabinoxylan (wheat)
6	Xyloglucan (tamarind seed)
7	MLG Lichenan, β-glucan (1-3),(1-4)-β-D-glucan)
8	β-glucan (yeast), (1-6),(1-3)-β-D-glucan)
9	β-glucan (oat), (1-3),(1-4)-β-D-glucan)
10	β-glucan (barley flour), (1-3),(1-4)-β-D-glucan)
11	β-glucan (Euglena gracillis), (1-3),(1-4)-β-D-glucan)
12	Carboxymethyl cellulose (CMC 4M)
13	Hydroxymethyl cellulose
14	Hydroxyethyl cellulose
15	Hydroxypropyl cellulose
16	2-hydroxyethyl cellulose
17	Methyl cellulose
18	Pachyman, (1-3)-β-D-glucan)
19	Pullulan, (1-6),(1-4)-α-D-glucan
20	Laminarin
21	Arabinogalactan, Type II (AGP)
22	Locust bean gum, galactomannan rich gum
23	Gum Guar
24	Gum karaya
25	Gum tragacant
26	Gum Ghatti (Indian gum)
27	Xanthane gum (Rhodigel 80)
28	Xanthane gum (Rhodigel TSC)
29	Gum Arabic
30	Lime pectin DE: 81% (E81)
31	Lime pectin DE: 15% (B15)
32	Lime pectin DE: 43% (B43)
33	Lime pectin DE: 64% (B64)
34	Lime pectin DE: 71% (B71)
35	Lime pectin DE: 11% (F11)
36	Lime pectin DE: 31% (F31)
37	Lime pectin DE: 58% (F58)
38	Lime pectin DE: 76% (F76)
39	Lime pectin DE: 16% (P16)
40	Lime pectin DE: 32% (P32)
41	Lime pectin DE: 46% (P46)
42	Lime pectin DE: 60% (P60)
43	Lime pectin DE: 66% (P66)
44	Lime pectin DE: 76% (P76)
45	Sugar beet pectin with DE 62% &DA 30%
46	Sugar beet arabinan
47	Linear arabinan
48	Pectic galactan, (1-4)-β-D-galactose polymer
49	RGI (soybean)
50	RGI (potato)
51	Lime pectin DE: 0% (E0)
52	Lemon pectin
53	Apple pectin
54	CP Kelco pectin
55	Sigma esterified citrus pectin
56	Feruloylated pectin
57	Feruloylated arabinoxylan
58	α-(1-5)-L-arabinobiose
59	α-(1-5)-L-arabinotriose
60	α-(1-5)-L-arabinotetraose
61	α-(1-5)-L-arabinopentaose
62	α-(1-5)-L-arabinohexaose
63	α-(1-5)-L-arabinoheptaose
64	α-(1-5)-L-arabinooctaose
65	D-galactose
66	β-(1-4)-D-galactobiose
67	β-(1-4)-D-galactopentaose
68	6^1-α-D-galactosyl-β-(1-4)-D-mannobiose
69	6^1-α-D-galactosyl-β-(1-4)-D-mannotriose
70	6^1-α-D-galactosyl-β-(1-4)-D-mannobiose/mannotriose
71	(1-6^3,6^4)-α-D-digalactosyl-β-(1-4)-D-mannopentaose
72	D-mannose
73	β-(1-4)-D-mannobiose
74	β-(1-4)-D-mannotriose
75	β-(1-4)-D-mannotetraose
76	β-(1-4)-D-mannopentaose
77	β-(1-4)-D-mannohexaose
78	isoprimeverose, α-D-xylopyranosyl-(1-6)-D-glucose
79	Xyloglucan heptamer, XXXG~OH (Megazymes)
80	Xyloglucan heptamer, XXXG~OH (XGO7)
81	Xyloglucan heptamer, XLLG~OH (XGO9)
82	XG-oligosaccharide (XG14)
83	β-(1-4)-D-xylobiose
84	β-(1-4)-D-xylotriose
85	β-(1-4)-D-xylotetraose
86	β-(1-4)-D-xylopentaose
87	β-(1-4)-D-xylohexaose
88	Aldouronic acids 4^2-α-D-glucoronosyl-β-(1-4)-D-xylotetraose
89	Glucoronoxylan oligo (XU^{4}m^{2}XX)
90	Glucoronoxylan oligo (U^{4}m^{2}XX)
91	Cellobiose, β-(1-4)-D-glucobiose
92	Cellotriose, β-(1-4)-D-glucotriose
93	Cellotetraose, β-(1-4)-D-glucotetraose
94	Cellopentaose, β-(1-4)-D-glucopentaose
95	Cellohexaose, β-(1-4)-D-glucohexaose
96	(1-3),(1-4)-β-D-glucotriose (Mlg3a)
97	(1-3),(1-4)-β-D-glucotriose (Mlg3b)
98	(1-3),(1-4)-β-D-glucotetraose (Mlg4a)
99	(1-3),(1-4)-β-D-glucotetraose (Mlg4b)
100	(1-3),(1-4)-β-D-glucotetraose (Mlg4c)
101	Laminaribiose, β-(1-3)-D-glucobiose
102	Laminaritriose, β-(1-3)-D-glucotriose
103	Laminaritetraose, β-(1-3)-D-glucotetraose
104	Laminaripentaose, β-(1-3)-D-glucopentaose
105	Laminarihexaose, β-(1-3)-D-glucohexaose
106	Maltose, α-(1-4)-D-glucobiose
107	Maltotriose, α-(1-4)-D-glucotriose
108	Maltopentose, α-(1-4)-D-glucopentaose
109	Maltohexaose, α-(1-4)-D-glucohexaose
110	Maltotetraose, (1-6), (1-4)-α-D-glucotetraose
111	Maltoheptaose, (1-6), (1-4)-α-D-glucoheptaose
112	N-acetyl-2-deoxy-2-amino-D-glucose
113	Diacetyl-chitobiose
114	Triacetyl-chitotriose
115	Tetraacetyl-chitotetraose
116	Pentaacetyl-chitopentaose
117	Hexaacetyl-chitohexaose
118	Lactose, D-galactosyl-β-(1-4)-D-glucose
119	D-glucose
120	4^2,6^2-α-D-digalactosyl-β-(1-4)-D-galactobiose
121	6^2-β-D-galactosyl-β-(1-4)-D-galactotriose
122	6^2-α-D-galactosyl-β-(1-4)-D-galactotriose
123	α-(1-5)-L-arabinobiose, feruloylated
124	α-(1-5)-L-arabinotriose, feruloylated
125	β-(1-4)-D-galactobiose, feruloylated
126	RGI backbone (chem. synth. Rha-GalA-Rha-GalA-Rha-GalA)
127	BSA
B	Blank

Table A2. 4 Overview of the glycan-binding properties of the mammalian Fbs proteins family (tested on CFG glycan arrays by Henry Paulson Lab, University of Michigan Medical School, Ann Arbor, Michigan USA).

Glycan #	IUPAC Glycan Name	Mean RFU	SD	S/N
FBG1 tested on printed array_v1 (Jan 2006)				
141	Man5_9mix	5579	1424	3,92
144	Manα1-2Manα1-6(Manα1-3)Manα1-6(Manα2Manα2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-N	4866	990	4,91
140	Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4 GlcNAcβ-N	4562	1052	4,34
142	Manα1-6(Manα1-3)Manα1-6(Manα2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-N	4530	1204	3,76
145	Manα1-2Manα1-2Manα1-3(Manα1-2Manα1-3(Manα1-2Manα1-6)Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-N	3801	1835	2,07
143	Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-N	3334	1227	2,72
190	Neu5Acα2-6GalNAcα–Sp8	1181	1872	0,63
2	AGP-A	819	1526	0,54
17	**(4S)**Galβ1-4GlcNAcβ-Sp8	770	1483	0,52
55	Galβ1-3Galβ–Sp8	628	1083	0,58
FBG2 tested on printed array_v2.1 (Feb 2007)				
199	Man5_9mix	43313	5669	7,64
198	Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4 GlcNAcβ-N	41666	6731	6,19
50	Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4 GlcNAcβ-Gly	40992	2433	16,85
4	Ceruloplasmin	38013	6249	6,08
192	Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Asn	37939	6600	5,75
193	Manα1-2Manα1-6(Manα1-3)Manα1-6(Manα2Manα2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Asn	35702	16156	2,21
26	**(3S)(6S)**Galβ1-4**(6S)**GlcNAcβ-Sp0	35681	6559	5,44
197	Manα1-6(Manα1-3)Manα1-6(Manα2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Asn	33730	5713	5,90
194	Manα1-2Manα1-2Manα1-3(Manα1-2Manα1-3(Manα1-2Manα1-6)Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Asn	33535	14934	2,25
6	Transferrin	33382	11302	2,95
5	Fibrynogen	32042	14826	2,16
1	AGP	31755	8247	3,85
35	**(3S)**Galβ1-4**(6S)**GlcNAcβ-Sp8	30405	13114	2,32
45	**(6S)**Galβ1-4**(6S)**Glcβ-Sp8	29387	7362	3,99
39	**(4S)(6S)**Galβ1-4GlcNAcβ-Sp0	27019	11810	2,29
30	**(3S)**Galβ1-4**(6S)**Glcβ-Sp8	25712	9081	2,83
FBG4 tested on printed array_v2.1 (Feb 2007)				
4	Ceruloplasmin	36198	5578	6,49
26	**(3S)(6S)**Galβ1-4**(6S)**GlcNAcβ-Sp0	35002	6987	5,01
5	Fibrynogen	34666	8675	4,00
6	Transferrin	34258	10160	3,37
45	**(6S)**Galβ1-4**(6S)**Glcβ-Sp8	19795	3502	5,65
3	AGP-B (AGP ConA bound)	19116	3519	5,43
1	AGP	17319	1666	10,40
23	β-GlcN(Gc)-Sp8	15805	2288	6,91
29	**(3S)**Galβ1-4**(6S)**Glcβ-Sp0	14603	4221	3,46
2	AGP-A (AGP ConA flowthrough)	13946	2808	4,97
113	Galα1-6Glcβ-Sp8	13748	9041	1,52
35	**(3S)**Galβ1-4**(6S)**GlcNAcβ-Sp8	13525	2145	6,31
164	GlcNAcβ1-3Galβ1-4GlcNAcβ-Sp0	12539	6572	1,91
41	6-H2PO3Manα-Sp8	12163	1425	8,54
184	GlcAb-Sp8	11533	5541	2,08
8	α-D-Glc-Sp8	11476	3264	3,52
55	Fucα1-2Galβ1-3GalNAcβ1-3Galα-Sp9	11360	1558	7,29
7	α-D-Gal-Sp8	10684	5526	1,93
171	(GlcNAcβ1-4)6β-Sp8	10657	4393	2,43
38	**(3S)**Galβ-Sp8	10214	3613	2,83
27	**(3S)(6S)**Galβ1-4GlcNAcβ-Sp0	10015	2396	4,18
FBG5 tested on printed array_v2 (Feb 2007)				
4	Ceruloplasmin	47138	986	47,81
6	Transferrin	35459	6115	5,80
1	AGP	24908	3201	7,78
3	AGP-B (AGP ConA bound)	24283	2418	10,04
2	AGP-A (AGP ConA flowthrough)	23266	5185	4,49
26	**(3S)(6S)**Galβ1-4**(6S)**GlcNAcβ-Sp0	19160	6144	3,12
45	**(6S)**Galβ1-4**(6S)**Glcβ-Sp8	5702	838	6,80
13	α-L-Rha-Sp8	5323	742	7,17
30	**(3S)**Galβ1-4**(6S)**Glcβ-Sp8	5128	573	8,95
12	α-L-Fuc-Sp9	4556	2725	1,67
55	Fucα1-2Galβ1-3GalNAcβ1-3Galα-Sp9	4380	947	4,63
5	Fibrynogen	4123	722	5,71
66	Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ-Sp0	4068	457	8,90
FBG3 tested on printed array_v2.1 (Feb 2007)				
no glycan binding				

high affinity = mean RFU > 3 * average mean RFU

lower affinity = average mean RFU <= mean RFU < 3 * average mean RFU

Glycan structures decorating the Fbs-interacting glycoproteins (present on glycan arrays) are shown in Table A2.5.

Table A2. 5 Glycan structures decorating the Fbs-interacting glycoproteins (present on the CFG glycan arrays).

Ceruloplasmin	Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3(Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-N
	Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3(Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-N
	Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6(Neu5Acα2-6Galβ1-4GlcNAcβ1-2(Neu5Ac2-6Galβ1-4GlcNAcβ1-4)Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-N
	Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6(Neu5Acα2-6Galβ1-4GlcNAcβ1-2(Neu5Ac2-6Galβ1-4Fucα1-3GlcNAcβ1-4)Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-N
	Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6(Neu5Acα2-6Galβ1-4GlcNAcβ1-2(Neu5Ac2-3Galβ1-4GlcNAcβ1-4)Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-N
Transferrin	Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3(Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-N
	Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6(Neu5Acα2-6Galβ1-4GlcNAcβ1-2(Neu5Ac2-6Galβ1-4Fucα1-3GlcNAcβ1-4)Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-N
	Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-N
Fibrinogen	GalNAcβ1-4GlcNAcβ1-4Manα1-6(GalNAcβ1-4GlcNAcβ1-4Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-N–Sp1
	GlcNAcβ1-2Manα1-3(GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-N-SP1
	NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-N
	NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3(NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-N
	NeuAcα2-8NeuAcα2-(3,6)Galβ1-4GlcNAcβ1-2Manα1-3(NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-N
	NeuAcα2-8NeuAcα2-8NeuAcα2-(3,6)Galβ1-4GlcNAcβ1-2Manα1-3(NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-N
a1-Acid glycoprotein (AGP)	Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3(Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-N
	±Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6(±Neu5Acα2-6Galβ1-4GlcNAcβ1-2(±Neu5Ac2-6Galβ1-4(±Fucα1-3)GlcNAcβ1-4)Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-N
	±Neu5Acα2-6Galβ1-4GlcNAcβ1-2(±Neu5Acα2-3/6Galβ1-4GlcNAcβ1-6)Manα1-6(±Neu5Acα2-6Galβ1-4GlcNAcβ1-2(±Neu5Acα2-3/6Galβ1-4(±Fucα1-3)GlcNAcβ1-4)Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-N
	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2(Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-6)Manα1-6(±Neu5Acα2-3/6Galβ1-4GlcNAcβ1-2(±Neu5Acα2-3/6Galβ1-4GlcNAcβ1-4)Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-N

Table A3. 1 Overview of primers used in qRT-PCR.

Target gene	Forward primer (5'-3')		Reverse primer (5'-3')	
F-box-Nictaba gene *(At2g02360)*	evd786	TTGAGCTTGGGGAGTTCTTC	evd787	AGAGGATTTTAGCAGGTCGG
GALT1 (At1g26810)	evd1153	AGTGATGGATGCAAGGATGG	evd1154	GAGAGCGTTTGGTTTCTTGG
FUT13 (At1g71990)	evd1155	TTTCTATGCGCTCGACTCTG	evd1156	GAGCCGAATTTGCTACCATC
PP2A (*At1g13320)* – reference gene for data normalization	evd727	TCCGAGATCACATGTTCCAAACTC	evd728	CCGTATCATGTTCTCCACAACCG
TIP41 (At4g34270) – reference gene for data normalization	evd729	TGAACTGGCTGACAATGGAGTG	evd730	CATGAGCTTGGCATGACTCTCAC
UBC9 (At4g27960) – reference gene for data normalization	evd731	TCCTACTTCATGTAGCGCAGGAC	evd732	TCCTCCAGAATAAGGGCTATCCG
ARR5 (At3g48100) – positive control for BAP treatment	evd741	CCTGATTCTTTCGGCTTACAATTT	evd742	TGATCAGTCTTGGTTCTATCAGCAA
COR15A (At2g42540) – positive control for ABA and cold treatment	evd781	CAGTGAAACCGCAGATACATTGGG	evd782	GGCTTCTTTTCCTTTCTCCTCC
ERS1 (At2g40940) – positive control for ethephon treatment	evd813	GGTTTGTCGGGCTAATGG	evd814	ACCACTGCTACTGCTTGGAC
GAI (At1g14920) – positive control for GA_3 treatment	evd743	AATGAATTGATCTGTTGAACCGG	evd744	GGCTTCGGTCGGAAATCTATC
HsfA2 (At2g26150) – positive control for MG132 treatment	evd1095	GTGTTGAGGTTGGGCAATACG	evd1096	TTGCTGTTGCCTCAACCTAACTAC
Hsp70b (At1g16030) – positive control for heat treatment	evd735	ATGTATCAGGGTGGTGCTGCT	evd736	ACCTCTTCGATCTTGGGACCT
IAA1 (At4g14560) – positive control for IAA treatment	evd739	AGGACACAGAGCTTCGTTTGG	evd740	GTCGTTGTTCTTGCGCTTGT
JMT (At1g19640) – positive control for MeJA treatment	evd745	TATGTAAGCTCGCCACGATACGCT	evd746	AACACGATCAACCGGCTCTAACGA
PDF1.2 (At5g44420) – positive control for *B. cinerea* infection	evd788	AAGTTGTGCGAGAAGCCAAG	evd789	CCATGTTTGGCTCCTTCAAG
PR1 (At2g14610) – positive control for *Pseudomonas* infection	evd1019	GCTACGCAGAACAACTAAGAGG	evd1020	GCCTTCTCGCTAACCCACAT
RD29A (At5g52310) – positive control for mannitol and NaCl treatment	evd749	ATCACTTGGCTCCACTGTTGTTC	evd750	ACAAAACACACATAAACATCCAAAGT
WRKY70 (At3g56400) – positive control for SA treatment and *Pseudomonas* infection	evd811	CATGGATTCCGAAGATCACA	evd812	CTGGCCACACCAATGACAA

Table A3. 2 Overview of primers used in testing the SALK lines.

Target gene/sequence	Forward primer (5'-3')	Reverse primer (5'-3')[a]
Left border of the T-DNA insertion sequence	LBb1.3/P99 ATTTTGCCGATTTCGGAAC	-
Genomic primers for KO4 line (SALK_007866)	$LP_{(KO4)}$/evd1009 CAGGCAACGAATCGAGAGTAG	$RP_{(KO4)}$/evd1010 AAACTTCGCGATGTATGTTGG
Genomic primers for KO6 line (SALK_085735C)	$LP_{(KO6)}$/evd1011 AATCTCCATCCACCCATCTTC	$RP_{(KO6)}$/evd1012 GTAGACGCGTGGATTGTTCAC
ACT2 (At3g18780)	evd280 GGCTGGATTTGCTGGAGATGATGC	evd281 GTACGACCACTGGCATACAGGGA
full-length *F-box-Nictaba* (*At2g02360*) gene sequence	evd790 CACCATGGGGAGAAAACGCAGAG	evd791 TCAGAGGATTTTAGCAGGTCGG

Table A3. 3 Overview of all primers used in molecular cloning.

Target gene/sequence	Forward primer (5'-3')[a]	Reverse primer (5'-3')[a]
1806 nt promoter sequence of *At2g02360*	evd 555 AAAAAGCAGGCTTCGGCCATCTTTTTAACCATTGC	evd 556 AGAAAGCTGGGTGCGACGCTTGTATCTTCGTC
full-length *F-box-Nictaba* (*At2g02360*) gene sequence	evd1046 AAAAAGCAGGCTTCACCATGGGGAGAAAACGCAGA	evd1047 AGAAAGCTGGGTGTCAGAGGATTTTAGCAGGTCGG
attB1 and attB2 adaptor sites	evd 2 GGGGACAAGTTTGTACAAAAAAGCAGGCT	evd 4 GGGGACCACTTTGTACAAGAAAGCTGGGT
NptII (kanamycine resistance gene)	evd 463 GAACAAGATGGATTGCACGCAGG	evd 261 TCAGAAGAACTCGTCAAGAAGGCG
UidA (β-glucuronidase gene)	GUS-F AAAAAGCAGGCTTCGATTTGGAAACGGCAGAGAAGG	GUS-RV AGAAAGCTGGGTGTTTCTTGTTACCGCCAACGCG

[a] Nucleotides underlined are complementary to parts of the attB1 and attB2 gateway cloning sites.

Table A3. 4 Putative *cis*-acting regulatory elements identified with high frequency in the *At2g02360* promoter sequence by *in silico* analyses for identical motifs stored in the PLACE (part 1), PlantCARE (part 2) and AGRIS (part 3) databases.

Motif	Frequency	Description
PART 1 – PLACE database output		
-300 ELEMENT	3	enhancer for endosperm specific-expression of glutenin
2SSEEDPROTBANAPA	1	Important for napA promoter
AACACOREOSGLUB1	5	endosperm-specific expression
ABRERATCAL	1	Ca^{2+}-responsive element
ACGTATERD1	4	Expression of erd1, induced by drought stress
AMYBOX1	3	amylase box
ANAERO1CONSENSUS	7	motif in promoters of anaerobically induced genes
ANAERO3CONSENSUS	2	motif in promoters of anaerobically induced genes
ARFAT	2	response towards auxine
ARR1AT	29	ARR1 binding site
ASF1MOTIFCAMV	1	ASF-1 binding site
BIHD1OS	3	Binding site for transcription factor OsBIHD1
BOXIINTPATPB	4	Important for NCII promoters
BOXLCOREDCPAL	1	Core sequence of box-L motif
CAATBOX1	21	Tissue specific promoter element of legA gene in pea
CACTFTPPCA1	26	Key component of Mem1
CANBNNAPA	1	endosperm-specific expression

CATATGGMSAUR	2	response towards auxine
CCA1ATLHCB1	1	Response towards light
CCAATBOX1	3	motif in promoters of heat shock proteins
CGACGOSAMY3	3	motif in GC-rich regions of rice amylase genes
CGCGBOXAT	2	Calmodulin-binding domain
CIACADIANLELHC	1	Necessary for Lhc circadian expression in tomato
CURECORECR	4	Copper and oxygen responsive element
DOFCOREZM	26	Binding site for Dof proteins
DPBFCOREDCDC3	2	bZIP transcription factor, induced by ABA and embryo-specific
DRE1COREZMRAB17	1	response towards ABA
E2FCONSENSUS	1	E2F consensus sequence
EBOXBNNAPA	14	E-box
EECCRCAH1	2	Consensus motif for enhancer elements EE-1 and EE-2
ELRECOREPCRP1	1	response towards elicitors
ERELEE4	3	response towards ET
GAREAT	3	response towards GA
GATABOX	15	response towards light and tissue-specific expression
GT1CONSENSUS	26	GT-1 binding site in light-induced genes
GT1CORE	3	Involved in binding of GT-1 to box II
GT1GMSCAM4	8	Involved in pathogen and salt-induced gene expression
GTGANTG10	15	Involved in expression of the late pollen gene g10
HEXAMERATH4	2	motif in histon H4 promoter of *A. thaliana*
IBOXCORE	7	response towards light
INRNTPSADB	4	initiator in promoters of genes in tobacco lacking a TATA-box
LTRE1HVBLT49	1	Response towards low temperature
LTRECOREATCOR15	1	core of LTRE-1
MYB1AT	8	response towards drought
MYB1LEPR	1	Involved in defense regulated gene expression in tomato
MYB2CONSENSUSAT	3	response towards drought
MYBATRD22	1	response towards drought
MYBCORE	3	response towards water stress
MYBCOREATCYCB1	2	Involved in activation of receptor genes
MYBGAHV	3	response towards GA
MYBPLANT	1	MYB binding site
MYBPZM	2	MYB binding site
MYBST1	4	MYB binding site
MYCATERD1	2	response towards drought
MYCATRD22	2	response towards drought
MYCCONSENSUSAT	14	response towards drought
NODCON1GM	3	Noduline sequence
NODCON2GM	7	Noduline sequence
NTBBF1ARROLB	2	Tissue specific expression, response towards auxine
OSE1ROOTNODULE	3	Active in infected cells of root nodules
OSE2ROOTNODULE	7	Active in infected cells of root nodules
PALBOXAPC	1	Present in fenylalanine ammoniumlyase genes
POLASIG2	2	plant polyA signal
POLASIG3	3	plant polyA signal
POLLEN1LELAT52	10	Involved in pollen-specific activation of tomato genes
PREATPRODH	1	pro-osmolarity responsive element
PRECONSCRHSP70A	7	consensus sequence of the pro-osmolarity responsive element
PYRIMIDINEBOXHVEPB1	2	pyrimidine-box
PYRIMIDINEBOXOSRAMY1A	3	pyrimidine-box
QELEMENTZMZM13	1	Involved in enhancer activity
RAV1AAT	5	Binding site for transcription factors in *A. thaliana*
RBCSCONSENSUS	1	rbcS consensus sequence
REALPHALGLHCB21	6	Involved in phytochrome regulation
RHERPATEXPA7	1	Root hair-specific *cis*-element
ROOTMOTIFTAPOX1	8	motif in promoter of rolD gene
SEBFCONSSTPR10A	1	response towards auxine
SEF3MOTIFGM	3	SEF3 binding site (soybean embryo factor)
SEF4MOTIFGM7S	3	SEF4 binding site (soybean embryo factor)
SORLIP1AT	1	response towards light
SREATMSD	2	Sugar-repressive element
SURECOREATSULTR11	3	sulfur-responsive element
SV40COREENHAN	2	SV40 core enhancer
TAAAGSTKST1	7	TAAG-motif
TATABOX3	1	TATA-box
TATABOX5	2	TATA-box
TATCCAOSAMY	1	element present in α-amylase promoters
TBOXATGAPB	1	T-box in promoter of GAPB gene
TGTCACACMCUCUMISIN	1	enhancer involved in fruit-specific expression of cucumisin

TRANSINITDICOTS	1	Initiation codon for translation in dicots
TRANSINITMONOCOTS	1	Initiation codon for translation in monocots
UP2ATMSD	2	Up2 motif
VOZATVPP	2	VOZ-binding site involved in pollen development
WBOXATNPR1	4	W-box
WBOXHVISO1	4	W-box
WBOXNTCHN48	3	W-box
WBOXNTERF3	6	W-box
WRKY710S	10	W-box
PART 2 – PlantCARE database output		
G-box	1	response towards light
GT1 motif	4	response towards light
MRE	1	response towards light
TCCC motif	1	response towards light
TCT motif	2	response towards light
chs-CMA1a	2	response towards light
AE-box	2	response towards light
Box I	2	response towards light
GA motif	1	response towards light
GAG motif	1	response towards light
GATA motif	1	response towards light
LAMP-element	1	response towards light
Sp1	1	response towards light
as-2-box	1	response towards light
Box 4	1	response towards light
Gap-box	1	response towards light
Box-W1	1	Elicitor responsive element
ELI-box3	1	Elicitor responsive element
GARE motif	1	response towards GA
P-box	1	response towards GA
TGA-element	3	response towards auxine
CGTCA motif	1	MeJA-responsive element
TGACG motif	1	MeJA-responsive element
TCA-element	2	response towards SA
HSE	1	Heat stress element
LTR	1	Involved in cold response
MBS	2	Response towards drought stress
ARE	3	Essential for anaerobic induction
TC-rich repeats	1	Involved in defense and stress response
O2-site	1	Involved in zein metabolism
Unnamed_6	1	SEF4 binding site
CCAAT-box	1	MYBHv1 binding site
Skn-1_motif	2	Involved in endosperm-specific expression
CAAT-box	36	Promoter element
TATA-box	30	Promoter element
CCGTCC-box	1	Involved in meristem specific activation
circadian	1	Involved in expression of circadiana genes
A-box	1	Function unknown
AAGAA-motif	3	Function unknown
Box E	1	Function unknown
CTAG-motif	1	Function unknown
W-box	1	Binding site for WRKY transcription factors
PART 3 – AGRIS database output		
W-box promoter motif	1	Binding site for WRKY transcription factors
DPBF1 and 2 binding site motif	1	Binding site for bZIP transcription factors
MYB4 binding site motif	1	Binding site for MYB4 transcription factor
LFY consensus binding site motif	1	Binding site for LFY transcription factor
BOXII promoter motif	1	Function unknown

Summary / Samenvatting

Plants have evolved diverse cellular mechanisms, which allow them to promptly sense and respond to external stress signals. In the past decade evidence has accumulated that plant defense against stress involves, among others, a specific group of inducible glycan-binding proteins, called lectins. These specialized proteins are synthesized by plants at very low (but physiologically relevant) concentrations after exposure to particular stress stimuli, such as drought, salt, wounding, microbial infection or insect herbivory. Therefore, it is suggested that by recognizing and binding specific glycan structures within plant cell, inducible plant lectins play a role in stress signaling pathways.

Another plant defense strategy against adverse environmental conditions is the ubiquitin-26S proteasome system (UPS). The UPS is a highly sophisticated machinery which controls most aspects of plant physiology by selective degradation of key regulatory proteins in the nucleus and cytoplasm of plant cells. The crucial components of this system are the F-box proteins, which are responsible for specific recognition of the target proteins destined for degradation. F-box proteins exhibit a typical bipartite structure and comprise an N-terminal F-box-domain and an C-terminal target-binding domain. They form the largest protein superfamily known with more than 800 putative representatives in *Arabidopsis thaliana*.

Most of the F-box proteins recognize substrates for degradation via protein-protein interactions. Nevertheless, several years ago, a group of putative carbohydrate-binding F-box proteins have been identified in plants, which contain a C-terminal domain homologous to Nictaba, the inducible nucleocytoplasmic lectin from tobacco plants. Consequently, it is hypothesized that F-box proteins with a lectin-like Nictaba domain could recognize specific carbohydrate structures present on glycoproteins and thereby would lead to the degradation of the latter protein. As such, plant proteins belonging to the so-called F-box-Nictaba family could presumably play a crucial role in plant stress physiology by integrating two defense-associated systems in plant cells: the UPS machinery and protein-carbohydrate interactions.

The F-box proteins with a Nictaba-related domain are widespread in the plant kingdom with more than 20 members in *A. thaliana.* The research of this PhD study was focused on the physiological relevance of one of these homologs from *A. thaliana* encoded by *At2g02360* and called F-box-Nictaba. The lectin-like domain of F-box-Nictaba shows the highest sequence similarity with the tobacco lectin and thus it is likely a functional carbohydrate-binding protein. Using glycan-binding assays, expression analyses and stress experiments with transgenic plants, the involvement of F-box-Nictaba in plant physiology and plant defense responses was investigated.

Chapter 1 presents a literature overview on plant lectins and their role in plant defense. Moreover, the chapter summarizes recent progress in the field of F-box-mediated protein degradation via the UPS and its significance for plant physiology.

In **Chapter 2** F-box-Nictaba is characterized at the molecular level. To experimentally corroborate the lectin activity of the F-box-Nictaba protein, the complete F-box-Nictaba sequence as well as its Nictaba-like domain were produced using the *Pichia pastoris* expression system, purified by affinity chromatography and characterized. Glycan microarray binding assays provided evidence for

carbohydrate-binding activity of both proteins, which showed virtually the same specificity, confirming that F-box-Nictaba is a functional lectin and can bind glycans via its C-terminal Nictaba domain. Screening of a glycan array containing predominantly carbohydrates of mammalian origin revealed reactivity towards *N*- and *O*-glycans containing *N*-acetyllactosamine (Galβ1-3GlcNAc and Galβ1-4GlcNAc), poly-*N*-acetyllactosamine ($[Galβ1\text{-}4GlcNAc]_n$), Lewis A (Galβ1-3(Fucα1-4)GlcNAc), Lewis X (Galβ1-4(Fucα1-3)GlcNAc, Lewis Y (Fucα1-2Galβ1-4(Fucα1-3)GlcNAc) and blood type B (Galα1-3(Fucα1-2)Galβ1-3GlcNAc) motifs. Analysis of arrays comprising plant-specific carbohydrates demonstrated that F-box-Nictaba preferentially recognizes β1-4-linked galactose oligomers and, with lower affinity, feruloylated α1-5-L-arabinobiose/triose glycans. Collectively, these results show that F-box-Nictaba exhibits glycan-binding activity preferentially directed against glycan structures containing a terminal galactose residue. What is more, these data revealed that the F-box-Nictaba specificity differs from that of Nictaba from tobacco, which specifically recognizes GlcNAc oligomers and high-mannose *N*-glycans.

Chapter 3 presents a detailed expression profiling of the gene encoding F-box-Nictaba using a combination of quantitative RT-PCR (qRT-PCR), β-glucuronidase (GUS) assay and *in silico* (co)expression analysis. It was demonstrated that *F-box-Nictaba* is continuously and stably expressed at a relatively low level throughout the lifecycle of plants grown under optimal conditions. However, *F-box-Nictaba* transcript levels significantly increase after specific stress treatments, including salicylic acid (SA), a plant hormone involved in defense responses, infection with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) as well as after heat stress. GUS histochemical staining experiments performed on transgenic *A. thaliana* plants indicated the preferential activity of the *F-box-Nictaba* promoter sequence in non-glandular leaf trichomes – structures associated with plant protection from adverse environmental conditions. Moreover, database searches revealed co-expression of *F-box-Nictaba* with genes involved in disease and plant defense responses.
Next, this chapter describes the application of selected stresses on transgenic *A. thaliana* plants with a knockout of *F-box-Nictaba* gene expression and plants overexpressing *F-box-Nictaba*. It was shown that plants overexpressing the F-box-Nictaba protein demonstrated higher expression of the *WRKY70* gene encoding a SA-related transcription factor and exhibited reduced disease symptoms after *Pst* DC3000 infection in comparison to wild type (WT) plants. Also, transgenic Arabidopsis plants with either reduced or enhanced F-box-Nictaba expression showed differential expression of the *Hsp70b* gene when compared to WT plants after heat stress. Finally, the *F-box-Nictaba* transcript levels themselves were also affected in the transgenic plants by heat stress conditions.

Based on the results of GUS assays, showing preferential *F-box-Nictaba* promoter activity in the trichomes of Arabidopsis plants, more detailed studies of trichome-specific expression were performed in **Chapter 4.** qRT-PCR experiments confirmed the pronounced *F-box-Nictaba* gene expression in the trichomes, while an immunodetection assay demonstrated significant amounts of F-box-Nictaba protein in these defense-related structures. Furthermore, we also analyzed the expression of genes encoding the enzymes β1,3-galactosyltransferase (GALT1) and α1,4-

fucosyltransferase (FUT13), which are required for the synthesis of Lewis A structures in Arabidopsis. It was shown that both genes necessary for the production of the glycan motif recognized by F-box-Nictaba on the glycan arrays are co-expressed in the trichomes.

Finally, **Chapter 5** discusses the significance of the research data and provides ideas for future studies. To conclude, all evidence gathered in the course of this PhD study shows that F-box-Nictaba is a functional lectin and suggests its involvement in plant defense responses directed towards stress stimuli of both biotic and abiotic origin. It is hypothesized that the role of F-box-Nictaba in plant stress signaling relies on the selective degradation of specific glycosylated proteins via the UPS. However, the putative substrates and the underlying mechanism of action remain to be elucidated. We believe that this research, altogether with further studies of F-box-Nictaba and other glycan-binding F-box proteins, will provide valuable insights into the complex network of plant stress responses and thus will significantly contribute to the development of more stress-resistant plants in the future.

Planten hebben verschillende mechanismen ontwikkeld om zeer snel en accuraat een antwoord te bieden op externe stress factoren. Zo is in de voorbije tien jaar duidelijk geworden dat o.a. een specifieke groep van induceerbare suikerbindende eiwitten, lectinen genaamd, een rol speelt bij de verdediging van een plant tegen stress. Deze lectinen worden door de plant in zeer lage, maar fysiologisch relevante hoeveelheden aangemaakt na blootstelling aan specifieke stress stimuli, zoals droogte, zoutstress, verwonding, pathogene infectie of insectenvraat. Er wordt verondersteld dat deze induceerbare plantlectinen een rol spelen in stress signaalpathways door middel van herkenning en binding van deze lectinen aan specifieke suikerstructuren in de plantencel zelf.
Een andere strategie die door planten toegepast wordt in stressafweer maakt gebruik van het ubiquitine-26S proteasoom systeem (ook UPS genaamd). Het UPS is een heel gesofisticeerd mechanisme dat bijna elke aspect in de plantenfysiologie controleert. De werking berust op de specifieke afbraak van belangrijke eiwitten, en dit zowel in de kern als in het cytoplasma van een plantencel. De cruciale molecules binnen dit systeem zijn de F-box-eiwitten. Deze eiwitten zijn verantwoordelijk voor de specifieke herkenning van doeleiwitten die moeten verwijderd worden. F-box-eiwitten hebben een typische opbouw bestaande uit een N-terminaal F-box domein en een variabel C-terminaal substraat-bindend domein. F-box-eiwitten vormen de grootste superfamilie van planteiwitten; in *Arabidopsis thaliana* zijn er momenteel meer dan 800 beschreven.
De meeste F-box-eiwitten herkennen hun substraat via eiwit-eiwit interacties. Enkele jaren geleden werd echter een groep van plant F-box-eiwitten ontdekt die een C-terminaal domein hebben dat grote gelijkenissen vertoont met Nictaba, het induceerbare nucleocytoplasmatische lectine uit tabaksplanten. Er wordt vermoed dat deze F-box-eiwitten met een Nictaba domein specifieke suikerstructuren die aanwezig zijn op glycoproteïnen kunnen herkennen en binden wat vervolgens kan leiden tot de afbraak van deze glycoproteïnen door het UPS. Op die manier kunnen deze plant F-box-Nictaba eiwitten een cruciale rol kunnen spelen in plantenafweer door het UPS mechanisme te combineren met eiwit-suiker interacties.
F-box-Nictaba eiwitten zijn wijdverspreid binnen het plantenrijk; momenteel zijn meer dan 20 van deze eiwitten geïdentificeerd in *A. thaliana*. In dit doctoraatsonderzoek werd gefocust op de fysiologische karakterisering van één van deze Arabidopsis homologen, namelijk het F-box-Nictaba eiwit dat gecodeerd wordt door het *At2g02360* gen. Binnen deze groep van homologe eiwitten vertoont het C-terminaal substraatbindend domein van dit specifieke F-box-Nictaba eiwit de hoogste sequentie-homologie met het tabakslectine Nictaba, waardoor dit F-box eiwit vermoedelijk een functioneel suikerbindend eiwit (lectine) is. Door middel van suikerbindings-assays, expressie-analyses en stress experimenten op transgene planten werd de functionaliteit van het geselecteerde F-box-Nictaba eiwit bestudeerd, met focus op de mogelijke rol in de fysiologie en stressafweer van Arabidopsis planten.

De literatuurstudie in **Hoofdstuk 1** bevat twee luiken. Eerst wordt er een overzicht gegeven van de huidige kennis omtrent plantlectinen en hun rol in stressafweer in planten. Daarna wordt een synopsis beschreven over plantaardige F-box-eiwitten, hun werking binnen UPS en hun rol binnen de fysiologie van planten.

In **Hoofdstuk 2** wordt de moleculaire karakterisering van F-box-Nictaba beschreven. De lectine-activiteit van dit Arabidopsis eiwit werd geanalyseerd op basis van recombinante eiwitten die geproduceerd en opgezuiverd werden uit de gist *Pichia pastoris*. Via de glycan array technologie kon aangetoond worden dat zowel het geselecteerde (recombinante) F-box-Nictaba eiwit als enkel het C-terminale domein (met grote homologie tot Nictaba) dezelfde specifieke suikerstructuren herkennen en binden. De resultaten uit deze assay bevestigen dus eerdere vermoedens dat het F-box eiwit lectine-activiteit vertoont en dat het suikers kan binden via zijn C-terminale Nictaba-homologe domein. Wanneer de eiwitten getest werden op 'glycan arrays' die hoofdzakelijk dierlijke (humane en muis) suikerstructuren bevatten, kon een specificiteit voor het plant F-box-eiwit vastgesteld worden voor zowel *N*- als *O*-glycanen gesubstitueerd met *N*-acetyllactosamine (Galβ1-3GlcNAc en Galβ1-4GlcNAc), poly-*N*-acetyllactosamine ($[Galβ1\text{-}4GlcNAc]_n$), Lewis A (Galβ1-3(Fucα1-4)GlcNAc), Lewis X (Galβ1-4(Fucα1-3)GlcNAc) en Lewis Y (Fucα1-2Galβ1-4(Fucα1-3)GlcNAc) motieven en voor suikerstructuren typerend voor bloedgroep B (Galα1-3(Fucα1-2)Galβ1-3GlcNAc). Wanneer de eiwitten getest werden op 'glycan arrays' die plantspecifieke suikerstructuren bevatten, kon een specificiteit voor het plant F-box-eiwit aangetoond worden voor β1-4 gekoppelde galactose oligomeren en, met lagere affiniteit, voor geferuloyleerde α1-5-L-arabinose/triose suikerstructuren. Zodoende kan besloten worden dat F-box-Nictaba een specifieke suikerbindende activiteit vertoont voor suikerstructuren die terminaal een galactose unit bevatten. Ondanks de hoge sequentie-homologie met het tabakslectine is de lectine-activiteit van dit F-box-Nictaba eiwit dus sterk verschillend van dit van Nictaba uit tabak, dat zelf specifiek bindt met GlcNAc oligomeren en hoog-mannose N-glycanen.

In **Hoofdstuk 3** wordt een gedetailleerde expressie-analyse beschreven voor het Arabidopsis gen *At2g02360* dat codeert voor het F-box-Nictaba eiwit, aan de hand van een kwantitatieve RT-PCR (qRT-PCR), een β-glucuronidase (GUS) assay en een in silico (co)expressie-analyse. Hierbij kon aangetoond worden dat F-box-Nictaba continu tot expressie wordt gebracht, in relatief lage hoeveelheden, gedurende de hele ontwikkeling van Arabidopsis planten die gecultiveerd werden in optimale omstandigheden. De transcript levels voor dit gen werden echter significant verhoogd wanneer de planten behandeld werden met specifieke stress factoren, zoals een behandeling met het (stress-responsieve) plantenhormoon salicylzuur, infectie met *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) en hittestress. Histochemische GUS experimenten toonden aan dat de promoter van het F-box-Nictaba gen voornamelijk actief is in een speciaal type van trichomen aanwezig op bladeren van Arabidopsis planten. Trichomen zijn specifieke cellulaire structuren die geassocieerd worden met de defensieve respons van de plant. In silico analyses gaven dan weer bijkomend aan dat F-box-Nictaba samen tot expressie komt met andere genen betrokken in ziekte- en stress-afweerrespons.

In ditzelfde hoofdstuk worden ook stressexperimenten beschreven met transgene *A. thaliana* planten die ofwel geen F-box-Nictaba meer tot expressie brengen (knockout lijnen) of net meer F-box-eiwit aanmaken (overexpressie-lijnen). Hieruit bleek dat in vergelijking met wild type planten de overexpressie-lijnen naast een hogere expressie van F-box-Nictaba ook een hogere expressie

vertonen van het WRKY70 gen, dat codeert voor een SA-gerelateerde transcriptiefactor. Deze lijnen gaven ook minder ziektesymptomen bij een infectie met de Pseudomonas stam (*Pst* DC3000) in vergelijking met wild type planten. Alle geteste transgene lijnen hadden ook een verschillend expressie-patroon voor zowel het Hsp70b gen als het gen voor F-box-Nictaba na een behandeling met hittestress in vergelijking met wild type planten.

Omdat de GUS assays wezen op een specifieke promoteractiviteit van het F-box-Nictaba gen in trichomen van Arabidopsis planten, werd een uitgebreidere expressie-analyse uitgevoerd voor dit gen in deze typische celstructuren. De resultaten van deze analyse worden beschreven in **Hoofdstuk 4**. De verhoogde expressie van F-box-Nictaba in blad trichomen kon bevestigd worden op basis van zowel qRT-PCR experimenten als van biochemische analyse-technieken (immunodetectie). Verder kon via qRT-PCR ook aangetoond worden dat de genen die coderen voor de synthetische enzymen noodzakelijk voor de aanmaak van de Lewis A suikerstructuur (d.i. de suikerstructuur die *in planta* door F-box-Nictaba kan worden gebonden) in Arabidopsis (zijnde β1,3-galactosyltransferase (GALT1) en α1,4-fucosyltransferase (FUT13)) ook opgereguleerd worden in trichomen.

Tot slot kan men in **Hoofdstuk 5** een uitvoerige discussie terugvinden die reflecteert over alle resultaten uit dit doctoraatsonderzoek. Verder worden enkele ideeën aangereikt om dit onderzoek verder uit te breiden in de toekomst. Samengevat kan men stellen dat werd aangetoond dat het geselecteerde F-box-Nictaba eiwit uit *A. thaliana* een functioneel lectine is, dat een rol kan spelen in de afweerrespons van de plant tegen stress stimuli van zowel biotische als abiotische oorsprong. Hoewel verondersteld wordt dat F-box-Nictaba functioneert in een UPS-gerelateerd mechanisme, blijft het totnogtoe onduidelijk welke de substraten (lees glycoproteïnen) zijn waarmee F-box-Nictaba interageert en binnenbrengt in het UPS voor afbraak. Onze onderzoeksresultaten met betrekking tot de functie van F-box-Nictaba eiwitten verschaffen waardevolle inzichten in het complexe netwerk van stress responsen dat bestaat in planten en kunnen bijdragen tot de verdere ontwikkeling van resistente gewassen.

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Karolina Stefanowicz,
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Curriculum vitae

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Publications

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- **Stefanowicz K**, Lannoo N, Proost P, Van Damme EJM (2012). Arabidopsis F-box protein containing a Nictaba-related lectin domain interacts with *N*-acetyllactosamine structures. FEBS Open Bio 2, 151–158.

- Van Hove J, **Stefanowicz K**, De Schutter K, Eggermont L, Lannoo N, Al Atalah B, Van Damme EJM (2014). Transcriptional profiling of the lectin ArathEULS3 from *Arabidopsis thaliana* towards abiotic stresses. J Plant Physiol 171, 1763-1773.

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Conferences

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- Van Damme EJM, Delporte A, Schouppe D, Van Hove J, Fouquaert E, Al Atalah B, **Stefanowicz K**, Lannoo N (2011). Role of nucleocytoplasmic lectins in plant cell signaling. 21st International Symposium on Glycoconjugates. 21-26 August, Vienna, Austria.

- **Stefanowicz K**, Lannoo N, Van Damme EJM (2011). Distinct glycan binding properties within the family of plant proteins containing a Nictaba-like lectin domain. 22nd Joint Glycobiology Meeting. 27-29 November, Lille, France.

- **Stefanowicz K**, Lannoo N, Van Damme EJM (2012). Role of a carbohydrate-binding F-box protein from *Arabidopsis thaliana* in plant defense against pathogens. FEBS Special Meeting on “Protein Quality Control and Ubiquitin Systems in Health and Disease”. 14-16 November, Kusadasi, Turkey.

- Maeda M, **Stefanowicz K**, Lannoo N, Kimura Y, Van Damme EJM. Occurrence of free *N*-glycans and expression of nucleocytoplasmic lectin(s) in tomato plants (2012). 23rd Joint Glycobiology Meeting. 25-27 November, Wageningen, The Netherlands.

- **Stefanowicz K**, Lannoo N, Van Damme EJM (2013). Glycan-binding F-box protein from *Arabidopsis thaliana* in plant defense responses. 65th International Syposium on Crop Protection. 21 May, Ghent, Belgium.

- **Stefanowicz K**, Lannoo N, Van Damme EJM (2014). Glycan-binding F-box proteins in plant defense. 25th Joint Glycobiology Meeting. 14-16 September, Ghent, Belgium.

with poster presentation

- Lannoo N, **Stefanowicz K**, Van Damme EJM (2012). Cross-talk among ubiquitin, SUMO and glycosylation in glycoprotein degradation in plants. Conference "Protein degradation pathways in health and diseases" hosted by the International Forum of Proteomics and Proteomics of Protein Degradation and Ubiquitin Pathways. 22-25 January, San Diego, California, USA.

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- Lannoo N, **Stefanowicz K**, Van Damme EJM (2012). Setting up an *in vitro* SUMOylation assay for a recombinant plant F-box protein consisting of a Nictaba-like lectin domain. 23rd Joint Glycobiology Meeting. 25-27 November, Wageningen, The Netherlands.

- Eggermont L, **Stefanowicz K**, Van Hove J, Van Damme EJM (2013). Expression analysis of Nictaba homologs in *Arabidopsis thaliana* subjected to abiotic and biotic stresses. 24th Joint Glycobiology Meeting. 24-26 November, Wittenberg, Germany.

without presentation

- 21st Joint Glycobiology Meeting. 7-9 November, Ghent, Belgium.

- International Symposium "Growth and Development of Roots" organized by Belgian Arabidopsis Root Network. 27 January, Louvain-la-Neuve, Belgium.