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Expression and nuclear interacting partners of the tobacco lectin

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Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences (Cell and Gene Biotechnology)
Expressie en nucleaire interactiepartners van het tabakslectine


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<td>amino acid</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AIG1</td>
<td>avrRpt2-induced gene 1</td>
</tr>
<tr>
<td>AP</td>
<td>affinity purification</td>
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<tr>
<td>ATP</td>
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<td>BiFC</td>
<td>bimolecular fluorescent complementation</td>
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<td>cyan fluorescent protein</td>
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<td>enhanced green fluorescent protein</td>
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<td>FITC</td>
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<td>FRAP</td>
<td>fluorescence recovery after photobleaching</td>
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<td>β-glucuronidase</td>
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<td>heterochromatin protein 1</td>
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<td>hexosamine signaling pathway</td>
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<td>JA</td>
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<td>JAME</td>
<td>methyl jasmonate</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LC</td>
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</tr>
<tr>
<td>LMB</td>
<td>leptomycin B</td>
</tr>
<tr>
<td>LRR</td>
<td>leucine rich repeat</td>
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<tr>
<td>MBT</td>
<td>malignant brain tumor</td>
</tr>
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<td>Description</td>
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</tr>
<tr>
<td>mC</td>
<td>cytosine methylation</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NB</td>
<td>nucleotide-binding</td>
</tr>
<tr>
<td>NE</td>
<td>nuclear envelope</td>
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<tr>
<td>Nictaba</td>
<td><em>Nicotiana tabacum</em> agglutinin</td>
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<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>NMR</td>
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<tr>
<td>NPC</td>
<td>nuclear pore complex</td>
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<td>OGA</td>
<td>O-GlcNAcase</td>
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<td>OGT</td>
<td>O-GlcNAc transferase</td>
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<tr>
<td>OPDA</td>
<td>12-oxo-phytodienoic acid</td>
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<td>plant homeodomain</td>
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<tr>
<td>PP2</td>
<td>phloem protein 2</td>
</tr>
<tr>
<td>PPI</td>
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<tr>
<td>PPL</td>
<td>pumpkin phloem lectin</td>
</tr>
<tr>
<td>PTM</td>
<td>post-translational modification</td>
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<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>SCF</td>
<td>Skp1-Cullin-1-F-box protein-Rbx1</td>
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<td>SEC</td>
<td>secret agent</td>
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<tr>
<td>Ser</td>
<td>serine</td>
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<td>SPR</td>
<td>surface plasmon resonance</td>
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<tr>
<td>TAP</td>
<td>tandem affinity purification</td>
</tr>
<tr>
<td>TET</td>
<td>ten eleven translocation</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll interleukin-1 receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMV</td>
<td>tobacco mosaic virus</td>
</tr>
<tr>
<td>Trp</td>
<td>tryptophan</td>
</tr>
<tr>
<td>TSS</td>
<td>transcription start site</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>UDP</td>
<td>uridine diphosphate</td>
</tr>
<tr>
<td>WGA</td>
<td>wheat germ agglutinin</td>
</tr>
<tr>
<td>Y2H</td>
<td>yeast two-hybrid</td>
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<td>YFP</td>
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Scope

In the last decade, a lot of research contributing to the multidisciplinary study of a group of stress-inducible nucleocytoplasmic lectins has been done. It was proposed that some of these lectins are involved in endogenous signaling events induced in the plant upon stress. But little is known about their precise function and mode of action. In this work, research is focused on the lectin expressed in leaves from *N. tabacum* after jasmonate treatment or insect herbivory, called Nictaba. Hence, in Chapter 1 a review is given on the current literature of what is known about the tobacco lectin. Since Nictaba was suggested to interact with histone proteins, also a brief introduction to epigenetics, focusing on the regulation of transcription and stress is included.

The first objective of this work was to characterize the tissue-specific expression of the tobacco lectin after jasmonate treatment during plant development. Therefore, in Chapter 2, the expression of Nictaba in the different plant tissues was followed during specific developmental stages using a GUS-reporter assay in transgenic Arabidopsis and tobacco plants. In addition, the Nictaba promoter sequence was analyzed in silico, to identify promoter-responsive elements.

Previously, it was shown that Nictaba is partly transferred into the nucleus due to the presence of a NLS. A second objective of this PhD research was to analyze the localization of Nictaba in the plant cell and to study nuclear transport of Nictaba. In Chapter 3, the subcellular localization of the lectin is assessed in different plant systems using different transformation techniques. The involvement of the NLS and the carbohydrate-binding activity of Nictaba in nuclear import were evaluated. Also the dynamic shuttling and mobility of the lectin inside the nucleus was analyzed.

Recently, a proteomics approach revealed that core histone proteins are the interaction partners for Nictaba. Moreover, this interaction most probably occurs through the O-GlcNAc modification present on the histone proteins. A third objective of the research was to explore the O-GlcNAc modification on plant histones in more detail. In Chapter 4, we study the presence of O-GlcNAc on nuclear proteins of tobacco and especially on histone proteins. Since cell cycle dependency was shown for animal O-GlcNAc modifications on histones, it was investigated whether O-GlcNAc levels also change during cell cycle progression in plant cells.

A fourth objective of the PhD was to corroborate the interaction between Nictaba and histones. In Chapter 5, this interaction was studied in detail. Plant histones were purified to examine their interaction with Nictaba in vitro. In addition, in vivo interaction studies were performed using microscopic techniques in an attempt to confirm the recognition and binding of Nictaba to tobacco histones.
Chapter 1

Introduction
Parts have been submitted

1.1 The tobacco lectin, prototype of the family of Nictaba-related lectins

1.1.1 Introduction

Lectins are a group of ubiquitous proteins of non-immune origin which bind reversibly to specific carbohydrate structures. These sugar-binding proteins are found in a wide variety of organisms ranging from plants to animals, viruses, bacteria and fungi (Lannoo and Van Damme 2010). For a long time, plant lectin research has concentrated on the purification and characterization of abundant lectins, especially found in seeds and vegetative storage tissues. Most of these highly expressed lectins are synthesized with a signal peptide and consequently are targeted via the secretory pathway into the vacuolar or extracellular compartment (Van Damme et al. 2008). At present, it is believed that these so called “classical” lectins combine a defense-related role against herbivorous insects or animals with a function as a storage protein (Van Damme et al. 2004). In the last decade, evidence has accumulated for the existence of a new class of low abundant lectins, which are only expressed after exposure of the plant to different stress factors and changing environmental conditions, and are therefore referred to as “inducible” lectins. In contrast to the classical lectins, these stress-related lectins are expressed in low concentrations and reside in the nuclear and cytosolic compartment of the plant cell (Van Damme et al. 2004, Van Damme et al. 2008).

All plant lectins known today can be classified in 12 families based on the sequence of the lectin polypeptides and the structure of their carbohydrate recognition domains (CRDs). Each CRD is characterized by its amino acid sequence, characteristic folding of the polypeptide and the structure of the binding site. Nevertheless, different CRDs can bind to similar glycan structures. According to the plant lectin classification system elaborated by Van Damme et al. (2008), all lectins that possess a similar carbohydrate binding domain should be grouped in one family.

In this chapter, we will focus on proteins belonging to the family of Nictaba-related lectins. The Nicotiana tabacum agglutinin or Nictaba was one of the first plant lectins that was classified as an “inducible lectin” (Chen et al. 2002). This review will describe the occurrence and distribution of Nictaba-related sequences in the plant kingdom. The carbohydrate-binding properties and biological activities of the proteins will be presented and discussed in view of their physiological role in plants.
Chapter 1: Introduction

1.1.2 Discovery of Nictaba

About a decade ago, Nictaba expression was first discovered in tobacco leaves of *N. tabacum* (cv Samsun NN) treated with methyl jasmonate (JAME) and jasmonate (JA). No lectin activity could be detected in leaves of untreated tobacco plants (Chen et al. 2002). Molecular cloning of the lectin yielded a cDNA sequence consisting of 498 nucleotides with a deduced amino acid sequence of 165 residues. Since this polypeptide lacks a signal peptide, translation most probably occurs on free polysomes in the cytoplasm. Protein sequence analysis also identified a lysine-rich region $^{102}$KKKK$^{105}$ which is a predicted classical monopartite nuclear localization signal (NLS) (Chen et al. 2002).

The tobacco lectin is a homodimer consisting of two identical non-covalently linked subunits of 19 kDa. Besides the addition of an acetyl group on the first methionine, no post-translational modifications are found on the protein. Nictaba is stable at neutral and basic pH but in contrast to many other plant lectins, the protein is quickly inactivated in an acidic environment (pH < 5). Furthermore, the lectin loses its activity at temperatures above 55 °C (Chen et al. 2002).

Nictaba is a genuine plant agglutinin since it can readily agglutinate human and animal red blood cells with a high preference for rabbit erythrocytes, the minimal concentration of the lectin for agglutination being 0.1 µg/ml, when trypsin-treated rabbit cells are used. Hapten inhibition assays revealed that N-acetylglucosamine (GlcNAc), GlcNAc oligomers and some animal glycoproteins (e.g. fetuin and ovomucoid) can inhibit this agglutination. GlcNAc oligomers are better inhibitors compared to the monosaccharide GlcNAc in the following order: chitotetraose, chitotriose and chitobiose. Surface plasmon resonance (SPR) experiments revealed that of all chito-oligosaccharides tested, $(\text{GlcNAc})_3$ showed the strongest interaction with Nictaba (Chen et al. 2002). These results have been confirmed by glycan array experiments using fluorescently labeled Nictaba. In addition, the glycan array also revealed interaction of Nictaba with high-mannose and bi-antennary N-glycans with terminal galactose (Gal), GlcNAc and N-acetylglactosamine (GlcNAc). Nictaba displays a significantly higher affinity towards N-glycans compared to the chito-oligosaccharides. A detailed study of the glycan array results indicated that the Nictaba binding site is most complementary towards $(\text{Man})_3\beta1-4\text{GlcNAc}\beta1-4\text{GlcNAc}\beta$-N-Asn (Lannoo et al. 2006b). To explain the binding of Nictaba to multiple sugar structures with different terminal carbohydrate residues, the hypothesis was put forward that Nictaba specifically interacts with the inner Man$_3$GlcNAc$_2$ core of the N-glycans which is identical for both complex and high-mannose N-glycans (Schouppe et al. 2010). Recently, saturation transfer difference NMR analyses have been performed to specifically study the interaction of glycan structures in the Nictaba binding site and to unravel the molecular basis for the specificity and affinity of this interaction (Gheysen 2011). These NMR studies confirmed that Nictaba
has the highest affinity towards the Man$_3$GlcNAc$_2$ core and chitotriose. In addition, the results suggest that at least two GlcNAc residues are necessary for the interaction with Nictaba, and the intensity of the binding increases with longer GlcNAc oligomers up to 3 residues. NMR results corroborated that Nictaba is interacting with the Man$_3$GlcNAc$_2$ core structure of N-glycans (Figure 1.1 A) and that the two GlcNAc residues are in closest proximity towards the Nictaba binding site (Figure 1.1 B).

![A and B](image)

**Figure 1.1** Schematic representation of the interaction between the Nictaba binding site and the Man$_3$GlcNAc$_2$ core (A) as deduced from saturation transfer difference NMR. Group epitope mapping was performed to show the proximity of the sugar residues from the Man$_3$GlcNAc$_2$ core in the binding site of Nictaba. The intensity of the red color reflects the closer proximity of Nictaba (B). Circles represent mannose residues whereas squares designate GlcNAc residues.

### 1.1.3 Nictaba and Cucurbitaceae phloem lectins are evolutionary related

BLAST sequence alignments revealed that the Nictaba sequence is evolutionary and structurally related to the sequences reported for different Cucurbitaceae phloem lectins (PP2). Furthermore, sequence alignments showed that the Nictaba sequence is much shorter (53 residues) at its N-terminus compared to the PP2 sequences, and also lacks the cysteine-rich C-terminal domain (5 residues) (Figure 1.2). The latter domain is required for Cucurbitaceae phloem lectins to interact with the phloem protein 1 (PP1) in the phloem exudates through multiple disulphide bridges (Chen et al. 2002). However, PP2 and Nictaba clearly share a common CRD, showing 24.7 % sequence identity and 41.2 % sequence similarity between Nictaba and the Nictaba homolog from melon (*Cucumis melo*). If we consider the CRD as the smallest unit exhibiting the ability to interact with carbohydrate structures, the Nictaba domain can be regarded as the basic CRD, whereas PP2 sequences consist of multiple domains, one of which corresponds to a Nictaba domain linked to additional N- and C-
terminal protein sequences. Hence, the Cucurbitaceae phloem lectins can be considered as a subgroup of the family of Nictaba-like proteins (Van Damme et al. 2008).

**Figure 1.2** Schematic representation of Nictaba and Cucurbitaceae phloem lectin sequences.

### 1.1.3.1 Characteristics of Cucurbitaceae phloem lectins

About three decades ago, strong agglutination activity was discovered in the phloem exudates of the cucurbit species pumpkin (*Cucurbita maxima*), cucumber (*Cucurbita sativus*) and melon (*Cucurbita melo*). These Cucurbitaceae phloem lectins were collectively called PP2. The pumpkin phloem lectin, renamed as PPL, is a 48 kDa dimer, consisting of two 26 kDa subunits linked by two disulphide bridges. This PPL is a structural protein which is highly abundant in phloem exudates (Sabnis and Hart 1978, Read and Northcote 1983). The PPL subunit is encoded by a 654 bp open reading frame that is translated into a 218 amino acid polypeptide lacking a signal peptide (Bostwick et al. 1992).

Physicochemical studies showed that this pumpkin lectin is unlike Nictaba stable up to 75°C (Narahari et al. 2010). Hapten inhibition assays for PPL yielded very similar results as for Nictaba in that only GlcNAc oligomers inhibited the agglutination of rabbit erythrocytes. Furthermore, the inhibition of the agglutination activity was also more successful for GlcNAc oligomers containing up to six GlcNAc residues (Narahari and Swamy 2010).

To this date, no glycan array results are available for PPL, but the carbohydrate binding properties of the PP2-like lectin PP2-A1 from Arabidopsis have been determined using the same glycan array setup as used for Nictaba. Similar to PPL and Nictaba, PP2-A1 recognizes chitin oligomers, preferentially chitotriose but also chitopentose and chitohexose albeit with lower affinity. Furthermore, PP2-A1 also binds with high-mannose N-linked glycans, with the common Man₃GlcNAc₂ core, and with the sialic acid derivative, 9-acyl-N-acetyllneuraminic sialic acid. (Beneteau et al. 2010).
1.1.3.2 Three-dimensional conformation of Nictaba and PPL

To analyze the secondary structural elements within Nictaba a circular dichroism (CD) spectrum and a hydrophobic cluster analysis (HCA) were made. Judging from the CD spectra, the Nictaba polypeptide folds into β-sheets (45%), β-turns and coils (55%), but has no α-helices. Furthermore, HCA results predicted that the putative NLS in the Nictaba sequence is located on an exposed loop connecting two strands of β-sheet and therefore accessible for NLS binding proteins (Chen et al. 2002).

A three-dimensional model of Nictaba was made based on the sequence homology between Nictaba and the carbohydrate binding module 22 of Clostridium thermocellum (Schouppe et al. 2010). This 3D-model is composed of two β-sheets of four and five antiparallel β-strands linked via extended loops (Figure 1.3 a). Within this model, the NLS is easily accessible since it is located at the top of a well exposed loop as suggested by the HCA results. The molecular surface of Nictaba consists of an extended electronegatively charged groove and an additional electronegative pocket, separated by a β-strand, most probably corresponding to the sugar binding site of the lectin (Figure 1.3 b).

The residues accounting for the electronegative character of the groove and the pocket are Glu138, Glu145 and Trp15, Trp22, respectively. To investigate the importance of these residues for the carbohydrate binding activity of the lectin, a mutational analysis was performed. Mutant lectin forms were expressed in Pichia pastoris and the sugar binding capacity of the recombinant proteins was evaluated (Schouppe et al. 2010). The authors demonstrated that the strongly conserved Trp15 residue is essential for sugar binding activity. They suggested that the carbohydrate binding activity of the tobacco lectin is maintained by the central electronegative groove consisting of conserved glutamic acid residues and carbohydrate interactions are stabilized by the association between the indole group of Trp15 and the pyranose ring of the glycan structure (Schouppe et al. 2010).
Figure 1.3 Molecular modeling of Nictaba. Ribbon diagram showing the β-sandwich organization of Nictaba (A). Exposed Lys residues are represented in cyan sticks, Glu residues in red sticks and Trp residues in yellow sticks. N and C correspond to the N- and C-terminal ends of the polypeptide chain, respectively. Mapping of electrostatic charges on the molecular surface of the modeled Nictaba (B). The putative carbohydrate-binding groove is delineated by a dotted white line and the location of important residues is indicated. Electronegatively and electropositively charged areas are colored red and blue, respectively, neutral regions are shown in white. Figure from Schouppe et al. 2010.

Similarly, a three-dimensional model of PPL was predicted by homology modeling using six templates, including the carbohydrate binding module 22 of C. thermocellum, and yielded 11.9% α-helix, 32.1% extended strand, 32.1% β-turn and 23.9% random coil (Figure 1.4). Interestingly, molecular docking showed that the binding pocket of PPL can accommodate up to three GlcNAc residues by water-mediated and van der Waals’ interactions. In the case of higher order chito-oligosaccharides only a triose unit was interacting with the lectin, while the remaining GlcNAc residues were more or less hanging out of the binding pocket, indicating that two triose units can interact with the sugar binding site of two different molecules of PPL (Narahari et al. 2011).
A comparison of the 3D-models for Nictaba and PPL reveals high similarity in the overall folding of the lectin polypeptides. The CRD of PPL consists of similar antiparallel β-strands and extended loops as observed in the 3D-model for Nictaba. Not surprisingly the N-terminal domain of PPL (absent in Nictaba) is completely different and folds as an α-helix structure.

Narahari and Swamy investigated the exposure and accessibility of the tryptophan residues of PPL using fluorescence spectroscopy. Quenching analyses in the presence and absence of chito-oligosaccharides were not significantly different, indicating that in contrast to Nictaba, Trp residues in PPL are not directly involved in the recognition and binding of carbohydrates (Narahari and Swamy 2009).
1.1.4 Distribution of Nictaba-like sequences in the plant kingdom

1.1.4.1 Occurrence of Nictaba-related sequences in different Nicotiana species

The distribution of Nictaba-related sequences within the genus Nicotiana was analyzed by screening 11 Nicotiana sections and 8 N. tabacum cultivars which were analyzed at genome level as well as at protein level. PCR amplification of sequences encoding Nictaba orthologs starting from genomic DNA was successful for 16 out of the 26 Nicotiana species/cultivars (Table 1.1) (Lannoo et al. 2006a). Upon sequencing, these genomic sequences were classified into three groups (i) containing a continuous complete ORF, (ii) containing one intron sequence or (iii) containing two intron sequences (Table 1.1). All sequences encoded Nictaba-like proteins consisting of 165 residues, showing at least 85% sequence identity to the Nictaba protein from N. tabacum cv Samsun NN.

At protein level, lectin activity was studied in methyl jasmonate-treated leaf tissues using agglutination assays and Western blotting. Lectin concentrations in leaf extracts from N. tabacum cultivars were remarkably higher compared to extracts from other Nicotiana sections. No lectin expression was detected in tobacco species/cultivars from which no Nictaba-related gene was amplified at DNA level (Table 1.1) (Lannoo et al. 2006a, Lannoo 2007b).
## Table 1.1 Overview of *Nicotiana* sections and *N. tabacum* cultivars tested for Nictaba-related sequences.

<table>
<thead>
<tr>
<th>Nicotiana classification</th>
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<th>Continuous ORF</th>
<th>One intron sequence</th>
<th>Two intron sequences</th>
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<tr>
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<td>+</td>
<td>Hav1</td>
<td></td>
<td>Sam2, Sam3</td>
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<tr>
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<td>Sam1</td>
<td>Sam4</td>
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<tr>
<td><em>N. sylvestris</em> Spegazzini &amp; Comes</td>
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<tr>
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</table>
1.1.4.2 Occurrence of Nictaba-related sequences in some completely sequenced genomes

A survey of the genome/transcriptome databases indicates that Nictaba orthologs are widespread among the Embryophyta but not common in other eukaryotes or prokaryotes. Besides *N. tabacum* and *Arabidopsis thaliana*, sequences containing a Nictaba domain have also been identified in many species throughout the plant kingdom. To get an idea about the distribution and evolution of the Nictaba domain, we have performed a detailed search in some completed plant genomes, representative for the different plant orders. In this selection, we included especially some plant species that are considered as model organisms. Our survey included rice as a monocot (*Oryza sativa*), as well as different dicots, such as *Solanum lycopersicum* (tomato), *Cucumis sativus* (cucumber). Furthermore we searched the genomes of the lower plants *Selaginella moellendorffii* and *Physcomitrella patens*. No Nictaba orthologs could be identified in *Chlamydomonas reinhardtii* or *Amborella trichopoda*.

Searches in the different transcriptome databases revealed that only a few orthologs consist of the Nictaba domain itself, for example the Nictaba lectin from tobacco (Figure 1.5). However, numerous sequences encode chimeric proteins comprising one or more Nictaba domain(s) fused to unrelated N- and C-terminal domains with (un)known function. The presence of C-terminal domains is rare and these domains consist of an unrelated and highly variable amino acid sequence (10-80 AA). In most Nictaba-related sequences an N-terminal domain with variable sequence precedes the Nictaba domain. These N-terminal sequences vary in length and amino acid composition, and often incorporate a protein domain with a known function. For example, the F-box domain is ubiquitous in plants and many F-box-Nictaba proteins can be found in all studied species for which the genome is complete. Other N-terminal domains preceding the Nictaba domain are the TIR (Toll/interleukin-1 receptor) domain in tomato and Arabidopsis, the AIG1 (avrRpt2-induced gene 1) domain in Arabidopsis and the protein kinase domain from rice. Furthermore, sequence alignment revealed that a few Nictaba orthologs from *O. sativa* contain 2 or 3 Nictaba domains in combination with N- and/or C-terminal domains. The latter results suggest that the Nictaba domain has been duplicated at one or more events during evolution.
Figure 1.5 Schematic overview of the different domain structures of Nictaba-like proteins in *N. tabacum*, *S. lycopersicum*, *A. thaliana*, *P. patens*, *S. moellendorffii*, *O. sativa* and *C. sativus*.

Based on the domain composition, the Nictaba orthologs can be classified into several types (Table 1.2). A first subdivision is made between the proteins containing one Nictaba domain and those containing multiple Nictaba domains. Within the group of proteins containing one Nictaba domain, type S0 represents the proteins that consist exclusively of a Nictaba domain. Putative proteins in which the Nictaba domain is preceded by an unrelated N-terminal domain are subdivided in different types depending on the length of the N-terminal sequence and the presence or absence of a C-terminal sequence. Type S1 consists of chimeric proteins with a short (<50 AA) N-terminal domain but no C-terminal sequence, whereas the type S2 has a short N-terminal as well as a C-terminal sequence. The absence or presence of the C-terminal sequence can also be found in proteins with a middle long (50-100 AA) or long (>100 AA) N-terminal domain, designated as type S3, S4, S5 and S6, respectively. Furthermore, there are several the chimeric proteins with a known N-terminal domain: TIR-Nictaba proteins (type T), AIG1-Nictaba protein (Type A), protein kinase-Nictaba protein (Type P) and F-box-Nictaba proteins (Type F). In the latter group, some proteins have additional unrelated short or middle long N-terminal sequences and/or a C-terminal sequence. The group of putative proteins containing multiple Nictaba domains is significantly smaller, at least within the species
selected for this study. The different proteins can be classified into type M1: a protein with a triple Nictaba domain which is preceded by a middle long N-terminal sequence. The second type M2 has a double Nictaba domain preceded by an F-box domain and followed by a C-terminal sequence. This classification system was elaborated based on the Nictaba orthologs found in *N. tabacum*, *S. lycopersicum*, *C. sativus*, *O. sativa*, *A. thaliana*, *S. moellendorffii* and *P. patens* but can easily be extended in case other types of Nictaba orthologs will be identified.

**Table 1.2** Summary of the different types of Nictaba orthologs and their occurrence in some plant species.

Analyses were done for *N. tabacum* (Nicta), *S. lycopersicum* (Solly), *A. thaliana* (Arath), *P. patens* (Phypa), *S. moellendorffii* (Selmo), *O. sativa* (Orysa) and *C. sativus* (Cucsa).

<table>
<thead>
<tr>
<th>Type</th>
<th>Number of genes in each species</th>
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<tr>
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<td>type M2</td>
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</table>

To unravel the evolutionary relationships among the sequences containing Nictaba domains, a phylogenetic tree (Figure 1.6) built using the Nictaba domain sequences for all Nictaba-related sequences detected in each of the species under study (Table 1.2). As shown in Figure 1.6, Nictaba orthologs from the same species are grouped together in one clade. In some cases, however, the Nictaba orthologs from different species are grouped by type. For instance, the F-box-Nictaba
proteins from rice, tomato, cucumber and Arabidopsis are grouped in one cluster of the dendrogram (Figure 1.6), which groups in a larger clade with the Nictaba sequences from the lower plants *Physcomitrella* and *Selaginella*.

**Figure 1.6** Phylogenetic analysis of Nictaba-like proteins. Phylogenetic tree constructed with the amino acid sequences of the Nictaba domains aligned using ClustalW. Bayesian phylogenetic inference via Markov chain Monte Carlo method was used to construct the tree (Huelsenbeck and Ronquist, 2001). For each protein, the prefix indicates the species from which it is derived, and the suffix indicates the type to which it belongs. In case of the proteins containing multiple Nictaba domains, all domains were incorporated in the analysis and the suffix A, B or C was added to distinguish the different lectin domains. All branches are drawn to scale as indicated by the scale bar and their length indicates the level of divergence among the sequences. The tree was visualized by FigTree ([http://tree.bio.ed.ac.uk/software/figtree/](http://tree.bio.ed.ac.uk/software/figtree/)).
Not surprisingly, the Nictaba sequence from tobacco and many of the Nictaba-like sequences from tomato cluster together. Most of the Nictaba sequences from Arabidopsis are found in two clusters of the dendrogram grouping mainly the sequences linked to F-box domains or the sequences linked to the AIG domain and the TIR domain. Both these clusters also have some sequences of the S type. Most Nictaba sequences from rice reside in one cluster, irrespective whether the sequence is of the S type or is linked to the protein kinase domain or the F-box domain. This cluster also contains the individual lectin domains of Nictaba-related sequences with multiple lectin domains from rice, indicating that these Nictaba domains are closely related. The first and the second Nictaba domain of the M1 and M2 type proteins cluster together. However, the third Nictaba domain of the M1 type is most similar to the Nictaba sequence from an F1 type protein from rice.

1.1.4.3 Chimeric proteins composed of Nictaba domains linked to unrelated domains

As mentioned before, in numerous sequences the Nictaba CRD domain is fused to a protein domain with a known function. Especially the F-box domain is highly represented among the chimeric proteins but also the AIG1 domain, the TIR domain and the protein kinase domain appear in combination with the Nictaba domain. The occurrence of chimeric lectins comprising a CRD domain and an unrelated protein domain is a common phenomenon that has been reported for different plant lectin families such as the Euonymus europaeus agglutinin family, the amaranthins and the ricin-B family (Van Damme et al. 2008).

Chimeric proteins with an F-box domain

F-box proteins are named after their conserved N-terminal F-box domain of 40-50 amino acid residues which was first discovered in cyclin-F (Bai et al. 1996) and which is coupled to various C-terminal interaction domains. This F-box domain is a motif that is used by the F-box proteins as a site for protein-protein interactions. Through their bipartite structure, F-box proteins can be assembled in a functional SCF-type (Skp1-Cullin-1-F-box protein-Rbx1) E3 ubiquitin ligase which comprises the target recognition subunit to attract proteins destined for degradation by the ubiquitin/proteasome machinery (Cardozo and Pagano 2004). With its N-terminal F-box domain, the F-box protein is bound to the Skp1 protein present in the SCF complex while the variable C-terminal interaction domain of the F-box proteins recruits target proteins. In this way, many F-box proteins are used by SCF complexes as substrate adaptors to mediate degradation of multiple regulatory proteins (Ho et al. 2006; Petroski and Deshaies 2005; Skaar et al. 2013). For more details, we refer to some recent publications (Finley 2009; Komander and Rape 2012).
The Arabidopsis F-box-Nictaba family groups approximately 30 members. They all share more than 90% and 40% sequence similarity in their F-box domain and Nictaba domain, respectively. The Nictaba domain in the F-box protein with Uniprot Acc. No. Q9ZVQ6, encoded by the gene At2g02360, shows the highest level of sequence similarity (64%) to the tobacco lectin (Lanoo et al. 2008). However, the overall sequence similarity between the Nictaba domain of Arabidopsis F-box-Nictaba proteins and the Nictaba protein from tobacco does not guarantee that this Nictaba domain shows carbohydrate binding activity. To this end, glycan-binding assays have been performed for the Arabidopsis F-box-Nictaba protein encoded by At2g02360 (Stefanowicz et al. 2012). These experiments illustrated that the selected Arabidopsis F-box-Nictaba protein exhibited a carbohydrate-binding activity towards N- and O-glycans with N-acetyllactosamine (LacNAc) (Galβ1-3GlcNAc and Galβ1-4GlcNAc) and poly-LacNAc structures as well as with Lewis A (Galβ1-3(Fucα1-4)GlcNAc), Lewis X (Galβ1-4(Fucα1-3)GlcNAc), Lewis Y (Fucα1-2Galβ1-4(Fucα1-3)GlcNAc and type-1 B motifs (Galα1-3(Fucα1-2)Galβ1-3GlcNAc). These motifs were clearly different from those bound by the tobacco lectin itself. Furthermore, the F-box-Nictaba protein showed only minor interaction with GlcNAc oligomers or with the inner core of high-mannose N-glycans, demonstrating that, despite the high sequence homology, the same structural Nictaba motif can accommodate different carbohydrate structures (Stefanowicz et al. 2012).

**Chimeric proteins with an AIG1 domain**

Infection with *Pseudomonas syringae*, carrying the avirulence Rpt2 gene (AvrRpt2), results in the up-regulation of the corresponding resistance gene (AvrRpt2 induced gene 1 or AIG1) in Arabidopsis plants (Reuber and Ausubel 1996). The *Pseudomonas* effector avrRpt2 stimulates the turnover of auxin/indole acetic acid proteins through which the auxin response is promoted to stimulate pathogenicity (Cui et al. 2013). AIG1 is a putative GTP binding protein that is suggested to participate in a series of defense mechanisms resulting in localized programmed cell death (Poirier et al. 1999; Rate et al. 1999). Until now, no information is available about the gene encoding the chimeric Arabidopsis protein consisting of the AIG1 domain linked to the Nictaba domain.

**Chimeric proteins with a TIR domain**

The N resistance protein from tobacco interacts with effector proteins after infection by the tobacco mosaic virus (TMV) and elicits hypersensitive responses (Whitham et al. 1994). Resistance proteins across species show structural similarity and contain a few well-conserved domains such as the leucine rich repeat (LRR) domain, the nucleotide binding (NB) domain, the Toll interleukin-1 receptor (TIR) domain and Toll-like receptor (TLR) domain (Kopp and Medzhitov, 1999; Marinon and Tschopp,
Chapter 1: Introduction

2005; Nurnberger, 2004). In mammals, the role and function of the TIR and TLR domains have been the subject of many studies and these show that these domains are components of the innate immune system. TLR domains are transmembrane receptors with the LRR domain at the N-terminus. Upon recognition of the pathogen-associated molecular pattern by the LRR domain, TLRs oligomerize and interact with adaptor molecules containing a TIR domain. Subsequently, different pathways are activated ultimately leading to the activation of transcription factors to regulate defense gene expression (Uematsu and Akira, 2006; Choe et al. 2005). In tobacco, the association between the N resistance protein and the TMV elicitor is mediated by the TIR domain of the N protein. Subsequent interaction between the avirulent protein and the NB and/or LRR domains results in the initiation of the defense mechanism in which the nuclear N protein is responsible for the signaling function of the R protein (Burch-Smith et al. 2007). To date, the Arabidopsis and tomato TIR Nictaba proteins have not been studied. Hence, further research is necessary to reveal the role of the Nictaba domain in the innate immune response against pathogens.

Chimeric proteins with a protein kinase domain

The group of protein kinases is a very extensive family of proteins that are defined as the enzymes that catalyze the reversible transfer of the γ-phosphate of adenosine triphosphate (ATP) to phosphorylate serine, threonine or tyrosine residues (Hanks and Hunter 1995). Lots of plant protein kinases have been identified as components of signal transduction cascades in response to light, plant hormones and nutrient deprivation (Stone and Walker 1995; Lethi-Shui and Shui 2012). All protein kinases have a 250-300 amino acid domain which mediates the phosphoryl transfer reaction and contains alternating subdomains (Hanks and Quinn 1991). At present, no information is available with respect to the gene or the corresponding putative protein kinase-Nictaba protein detected in rice.
1.1.5 Localization of plant proteins containing a Nictaba domain

Nictaba is located in the nucleus and the cytoplasm of tobacco parenchyma cells that have been subjected to jasmonate treatment. In these studies, immuno-cytochemistry was performed using an affinity-purified antiserum specifically directed against Nictaba (Figure 1.7). No fluorescence signal was detected in the vascular tissue, the chloroplast and the vacuole, or in non-treated tobacco leaves (Chen et al. 2002).

Figure 1.7 Immunolocalization of Nictaba in tobacco leaves. Cross section of a tobacco leaf of a control plant (a) and a plant treated with JAME for 5 days (b). Cytoplasm (c) surrounding the plastids, nucleus (n), plastids (pt), and vacuole (v) are indicated in a close-up (zoom-out) of (b) in (c) and (d). d) Shows a DAPI-staining of the section shown in (c). Bars represent 25 μm in (a) and (b), and 10 μm in (c) and (d). Figure from Chen et al. 2002.

The nucleocytoplasmic localization of Nictaba was confirmed in N. tabacum BY-2 suspension cells which were transiently transformed by particle bombardment with DNA encoding the fusion protein EGFP-Nictaba. Fluorescence signals were visible within the nucleus and the cytoplasm of the cell, whereas the nucleolus did not show any fluorescence. Interestingly, the localization patterns for the fusion protein in the nucleus changed at later time points after biolistic delivery (Lannoo et al. 2006b). Whereas initially (24h after particle bombardment) the fluorescence was evenly distributed over the nucleus, about 48 hours after transformation, it was more concentrated towards the periphery of the nucleus.

Analysis of the deduced amino acid sequence of the cDNA encoding Nictaba revealed the absence of a signal peptide and the presence of a putative NLS indicating that the lectin is synthesized on free polysomes and can be translocated into the nucleus. The functionality of the presumed NLS was examined by determining the subcellular localization of a fusion protein, consisting of EGFP covalently coupled with a mutant Nictaba sequence where the NLS $^{102}$KKKK$^{105}$ was altered into $^{102}$KTAK$^{105}$. After transient transformation into BY-2 cells, confocal microscopy showed that the mutant form of Nictaba located only to the cytoplasm and not to the nucleus of the cell, suggesting that the NLS sequence is necessary and sufficient to translocate Nictaba from the cytoplasm into the
nucleus. However, these BY-2 cells transformed with the construct encoding the NLS mutant exhibited a strong vesiculation and an increase in plasmolysis, possibly indicating the inception of cell death (Lannoo et al. 2006b).

As mentioned above, Nictaba expression was never observed in the vascular bundles. In contrast, Cucurbitaceae phloem lectin expression is developmentally linked to vascular differentiation. PPL proteins are synthesized within the companion cells of the phloem and subsequently are translocated to the sieve elements (Bostwick et al. 1992). It is known that sieve elements lose most of their cellular components such as nucleus, cytoskeleton, the vacuole, ribosomes and Golgi during ontogeny. The mature sieve elements only contain structural phloem specific proteins (P-proteins), mitochondria, smooth ER and sieve elements plastids (Froelich et al. 2011). PPL is present in the cytoplasm of the companion cells between numerous ribosomes but not in the mitochondria or the vacuole. After transportation into the mature sieve elements, PPL is again located in the cytoplasm in so-called P-protein bodies (Dannenhoffer et al. 1997).

1.1.6 Expression of the tobacco lectin

1.1.6.1 Jasmonate induced expression

Of all plant hormones tested (JA, 11-OH-JA, 12-OH-JA, 12-HSO₄-JA, 12-O-Glc-JA, JAME, 12-oxophytodienoic acid (OPDA), gibberellic acid, salicylic acid, indole-3-acetic acid, abscisic acid, 6-benzylaminopurine and ethephon) only JA, JAME and OPDA, a JA-precursor, enhanced Nictaba synthesis and expression (Lannoo et al. 2007a, Vandenborre et al. 2009a). Nevertheless, the expression levels for Nictaba in leaves are low, representing 1 to 30 mg/g fresh weight, depending on the leaf age and plant growth conditions. Optimal concentrations of JA and JAME to induce lectin activity were 100-150 µM and 50-100 µM, respectively. JAME treatment always yielded a significantly higher amount of lectin, compared to JA. The combination of different plant hormones with JAME did not result in an antagonistic or synergistic effect. Abiotic stress treatments (mechanical wounding, salt, heat, cold and UV-light) did not affect Nictaba expression (Lannoo et al. 2007a). In a Northern blot experiment the lectin mRNA was first detected 24 hours after jasmonate treatment and rapidly accumulated during the next 48 hours. Concomitantly the Nictaba protein accumulated starting from 24h after JAME induction and the lectin concentration reached its maximal levels after 72-96h (Chen et al. 2002).

Although all leaves of an adult tobacco plant express the lectin after JAME treatment, the highest lectin concentration is found in the newly expanded leaves. Lower lectin concentrations are observed in old and very young leaves, as well as in flower tissues (sepals and petals). No lectin activity was
detected in the stem (Chen et al. 2002). Recently, it was shown that also roots accumulate Nictaba after jasmonate treatment, albeit to a much lower extent compared to leaves (Delporte et al. 2011).

Interestingly, the jasmonate induction of Nictaba in tobacco leaves is systemic. When JAME is applied to a single leaf of the plant, Nictaba accumulation is not only observed in the treated leaf but also in the neighboring leaves. This systemic response is position-dependent and predominantly occurs in the apical direction. After removal of the JAME inducer, the Nictaba levels remained high for about ten days and then progressively decreased. After 6 weeks, the lectin was completely gone from the tobacco leaves of an adult plant (Lannoo et al. 2007a).

1.1.6.2 Insect herbivory induced expression

It was shown that insect herbivory by larvae of the cotton leafworm (Spodoptera littoralis) on a single leaf of a tobacco plant also enhanced Nictaba expression. Nictaba accumulation in the leaves was quantified during continuous feeding of the caterpillars and reached a maximal lectin content of 300 μg Nictaba/g FW after 15 hours of feeding (Lannoo et al. 2007a, Vandenborre et al. 2009a). The increase in lectin activity in tobacco leaves after insect feeding was accompanied by the accumulation of different jasmonates. These data indicate that the lectin synthesized in tobacco leaves after insect herbivory, is most probably the result of the activation of the jasmonate pathway (Vandenborre et al. 2009a). Similar to jasmonate induction, caterpillar feeding provokes a systemic response in the plant. After feeding of S. littoralis larvae for 15 hours on one leaf, all leaves of the plant clearly accumulate the lectin (Vandenborre et al. 2009a).

Since caterpillar-induced lectin expression presumably occurs through JA signaling, tobacco plants were also exposed to insects and pathogens known to enhance JA biosynthesis. Both arthropods S. littoralis and Tetramychus urticae induced Nictaba expression, while the necrotrophic pathogen Botrytis cinerea did not. JA signaling upregulates at least two groups of genes, a first group consisting of genes important for defense response against necrotic pathogens and a second group responsible for the expression of genes related to wounding and herbivory. These two groups are differentially regulated by the transcription factors MYC2 and EFT1. MYC2 represses the first group of genes and activates the second one, whereas EFT1 acts in the opposite way. Experiments performed by Vandenborre et al. (2009b) suggested that Nictaba is positively regulated by the MYC2 transcription factor.

In contrast to chewing insects (S. littoralis) and cell-content feeders (T. urticae), phloem-feeding insects such as aphids (Myzus nicotianae) and whiteflies (Trialeurodes vaporarium) did not affect Nictaba expression. Phloem-feeding insects caused only minor damage and induced a defense
response very similar to the one induced by salicylic acid signaling. The authors suggested that these insects manipulate the hormone signaling of the plant to suppress the JA defense response and consequently restrain Nictaba expression (Vandenborre \textit{et al.} 2009b). Furthermore, Nictaba accumulation upon insect herbivory was compared for a specialist insect such as \textit{Manduca sexta}, and a generalist insect such as \textit{S. littoralis}. The specialist insect clearly induced higher Nictaba levels compared to the generalist, possibly related to the higher JA levels generated upon \textit{M. sexta} attack (Vandenborre \textit{et al.} 2009b).

It has been suggested that PPL expression could be involved in the plant defense against fungi and bacteria (Read and Northcote 1983). The Arabidopsis PP2-A1 was shown to have no insecticidal activity using insect feeding assays with the pea aphid \textit{Acyrthosiphon pisum} and the green peach aphid \textit{Myzus persicae}. No significant differences in survival rate were recorded for nymphs fed with PP2-A1 compared to nymphs fed on a control diet. Although mid range concentrations of the lectin (between 125 and 250 µg/mL) caused a weight loss they were not affecting insect feeding behavior (Beneteau \textit{et al.} 2010).

1.1.7 Functional characterization of Nictaba

The physiological role of Nictaba within the cell is correlated with both the nucleocytoplasmic localization of the protein and its sugar binding specificity. In a first attempt to scrutinize the interaction of the tobacco lectin with nuclear proteins, Lannoo \textit{et al.} (2006b) performed a far Western blot experiment to check lectin binding to proteins extracted from nuclei purified from tobacco BY-2 cells. This preliminary experiment suggested that Nictaba is interacting with numerous nuclear proteins. The interaction was completely abolished by adding GlcNAc-oligomers, suggesting a lectin-carbohydrate interaction. Furthermore, the reactivity of Nictaba towards these proteins was reduced when N-glycans present on the nuclear proteins were enzymatically removed by PNGase F treatment. These results clearly demonstrated that Nictaba is interacting with endogenous glycosylated nuclear proteins in a sugar-inhibitable way (Lannoo \textit{et al.} 2006b).

In 2011, Schouppe \textit{et al.} applied a nuclear proteomics approach to identify putative interaction partners for Nictaba in a pool of nuclear proteins. Nuclear extracts from \textit{N. tabacum} cv Xanthi suspension cells were used in two complementary experiments. Both lectin affinity chromatography and pull-down assays revealed the interaction between Nictaba and histone proteins. The affinity of Nictaba for histones was confirmed by lectin affinity chromatography using a non-fractionated commercial histone preparation from calf thymus. Calf histone H2B was specifically retained on the Nictaba column and was eluted by 1 M GlcNAc. Moreover, the eluted proteins from the Nictaba column reacted with FITC (fluorescein isothiocyanate)-labeled Nictaba and with an antibody
specifically directed against O-GlcNAc modifications. Furthermore, MS analysis preceded by β-elimination identified the O-GlcNAc modification of calf histones H2B, H3.3 and H1 on residues Ser65, Thr80 and Thr101, respectively. These results suggest that Nictaba is interacting with the core histone proteins through their O-GlcNAc modification (Schouppe et al. 2011).

Since Nictaba expression is also enhanced after caterpillar attack, a role for Nictaba in plant defense was also suggested. The entomotoxic properties of the lectin towards the larvae of a generalist pest insect S. littoralis and a specialist M. sexta have been tested. Experiments have been performed using N. tabacum plants expressing the lectin gene under natural conditions upon herbivory, as well as N. attenuata plants in which the lectin gene was subjected to RNAi silencing. Furthermore N. attenuata plants, which lack the Nictaba gene, have been transformed with the Nictaba gene to create overexpression lines. Feeding of M. sexta larvae on transgenic N. attenuata plants, ectopically expressing Nictaba, displayed a significantly prolonged overall development time and a significantly lower pupal mass compared with larvae feeding on wild-type N. attenuata plants. Similar results were obtained in feeding experiments with S. littoralis using an identical setup, but the total mass gain reduction of M. sexta feeding on transgenic N. attenuata was only half of the reduction in mass gain for S. littoralis larvae, indicating different entomotoxic effects towards different Lepidopteran species. In line with the insecticidal activity of Nictaba, S. littoralis larvae performed better when feeding on transgenic N. tabacum plants in which Nictaba expression was silenced, compared to feeding on non-transformed N. tabacum plants expressing the lectin as a result of insect herbivory (Vandenborre et al. 2010).

The entomotoxic activity towards lepidopteran pest insects is presumably caused by the carbohydrate binding activity of Nictaba and its interaction with glycoconjugates present in the digestive tract of the insects. The midgut of caterpillars consists of a peritrophic membrane composed of e.g. chitin microfibrils, which are an obvious target for Nictaba. It was suggested that Nictaba could interfere with the organization of the chitin microfibrils. Moreover, it can be envisaged that Nictaba can bind to N-glycoproteins in the midgut forming large protein-Nictaba clusters, which in turn could interfere with transport and the activity of the digestive enzymes, instigating a growth inhibitory effect (Vandenborre et al. 2010).

1.1.8 Perspectives

Nictaba is a nucleocytoplasmic lectin which is only expressed after the plant has been subjected to certain stress conditions. Judging from the fact that only some specific jasmonate-related compounds as well as insect herbivory, which in turn switches on the jasmonate pathway, can enhance lectin expression, it is clear that Nictaba is part of a signaling cascade which involves
jasmonates. Part of the lectin is translocated into the nucleus by its internal NLS sequence. However, little is known about the influence of the carbohydrate binding activity in this process, the export of Nictaba and its dynamics. Inside the nucleus, Nictaba presumably interacts with histone proteins modified with O-GlcNAc, which is in good agreement with the high affinity of the lectin carbohydrate binding site towards GlcNAc oligomers. O-GlcNAcylation of histones was recently shown for mammals, but remains to be detected in plants. Also the interaction between Nictaba and core histones should be analyzed in planta.

1.2 Epigenetic regulation of transcription as a response to stress

1.2.1 Structural organization of eukaryotic chromatin

The primary role of the nucleus is to maintain a huge collection of genomic information. Because meters of DNA have to fit in a cell with a diameter up to a hundred thousand times smaller, the physical organization and compacting of chromosomal DNA is crucial (Woodcock and Ghosh 2010). The packing of genomic DNA is acquired by the abundant nuclear histone proteins. This complex of histones and DNA is termed chromatin. The nucleosome core particle is the repeating unit in chromatin and consists of an octamer containing two copies of each of the histones H2A, H2B, H3 and H4. A DNA sequence of 147 base pairs of DNA is wrapped in 1.65 superhelical turns around the protein core (Figure 1.8 A) (Luger 2006).

Figure 1.8 Space-filling model of the structure of the nucleosome based on X-ray crystallography. H2A is yellow, H2B is red, H3 is blue and H4 is green (A). Model of the 30 nm condensed chromatin fiber and beads on a string. The nucleosome is shown as an orange disk associated with one histone H1 molecule (B). Figure from Lodish et al. 2013.

Similar to protein, chromatin organization can also be categorized into different levels of structural hierarchy. The primary structure of chromatin was visualized by electron microscopy. After extraction using hypotonic buffers, chromatin revealed an open beads-on-a-string conformation
(Figure 1.8 B). The beads are the nucleosomes and the string is connecting or linker DNA. Here, a fifth histone, histone H1 is found, associated with the linker DNA. Using isotonic buffers, chromatin is already more compact and appears as 30-nm fibers (Woodcock and Ghosh 2010). It is currently believed that the 30-nm fibers are assembled in a zigzag higher-order structure, with contact between the odd and even numbered nucleosomes. Two nucleosome strands are wound into a two-start helix, which is a double helix similarly to a DNA double helix, except that the nucleosome helix is left handed rather than right handed (Schalch et al. 2005).

According to the higher order organization of chromatin, eukaryotic genomes can be subdivided into two distinct environments, euchromatin and heterochromatin. Euchromatin is characterized by an open chromatin structure, accessible for multiprotein complexes and containing most of the active genes. Heterochromatin consists of condensed regions containing centromeres and telomeres of chromosomes as well as transcriptionally inactive genes and is further subdivided into facultative and constitutive heterochromatin. Facultative heterochromatin corresponds to regions with genes whose expression is different during development and/or differentiation and then is silenced. Constitutive heterochromatin consists of permanently silenced genes (Bannister et al. 2011, Lodish et al. 2013).

1.2.2 The chromatin remodeling code

Epigenetics is defined as the study of reversible and heritable morphological variations not caused by DNA sequence changes. These variations are caused by altering the chromatin architecture. Remodeling chromatin structure can allow proteins involved in transcription or DNA replication to interact with specific genomic DNA segments. Several mechanisms, such as nucleosome occupation, incorporation of histone variants, DNA methylation and histone modifications are responsible for modifying the eukaryotic chromatin structure and create a chromatin remodeling code. These chromatin marks control transcription, DNA replication and DNA repair in response to development and environmental changes (Ha 2013).

Chromatin marks representing an accessible chromatin structure are found in euchromatin and typically are trimethylation of H3K4, H3K36 or H3K79 and a high level of acetylation. In contrast, the inaccessible heterochromatin is enriched in trimethylation of H3K9, K3K27 and H4K20 (Fuchs 2006, Kouzarides 2007, Bartova et al. 2008). Although histones and their modifications are strongly conserved among eukaryotes, the chromosomal distribution associated with the modifications can differ between groups of eukaryotes. For example, in Arabidopsis euchromatin is characterized by H3K4me1,2,3; H3K36me1,2,3; H3K9me3, H3K27me3 and H4K20me2,3. In heterochromatin H3K9me1,2; H3K27me1,2 and H4K20me1 are enriched. These distribution patterns are not preserved among plants (Fuchs 2006).
1.2.3 Histone modifications

Histone tails protruding from the nucleosomal surface are heavily subjected to post-translational modifications (PTM). Histone modifications can be classified into two major groups, those that alter the charge of the histone and therefore directly alter the chromatin organization, and those that create binding sites for other effector proteins (Berger 2007, Nelissen et al. 2007, Woodcock and Ghosh 2010, Bannister et al. 2011). One histone does not have all the possible modifications simultaneously, but the histones of a single nucleosome usually contain several of these modifications at the same time. The timing of the appearance of a set of modifications will depend on the signaling conditions within the cell. The particular combination of PTMs of chromatin constitutes the histone code that influences chromatin function by altering chromatin structure or, creating or removing binding sites for chromatin-associated proteins (Kouzarides et al. 2007, Lodish et al. 2013).

Acetylation

Lysine residues in the histone tails undergo reversible acetylation and deacetylation by two families of enzymes, histone acetyltransferases and histone deacetylases. This highly dynamic reaction neutralizes the positive charge of lysine, thereby weakening the electrostatic interactions between histones and DNA. Consequently, histone acetyltransferases are characterized as transcriptional co-activators whereas histone deacetylases, who stabilize the chromatin architecture by removing the acetyl-group, are transcriptional repressors (Wang et al. 2008, Bannister et al. 2011). Also in plants, histone acetylation is enriched at enhancer elements and specifically at gene promoters, and deacetylation is associated with silencing of repetitive transgenes (Fuchs 2006).

Phosphorylation

Addition and removal of a phosphate group is achieved by the alternate action of kinases and phosphatases and occurs on serines, threonines and tyrosines. Similar to acetylation, phosphorylation is highly dynamic and adds a negative charge to the histone backbone which influences chromatine architecture (Bannister et al. 2011). Phosphorylation has been linked with different functions such as activating transcription, apoptosis and DNA repair (Fuchs 2006). Phosphorylation is also linked with chromosome condensation/segregation and marks their progress through the cell cycle. For example, phosphorylation of serine residues in histone H3 is initiated in the late G2-phase and continues up to the end of mitosis, in parallel with chromatin condensation (Houben et al. 2007, Nelissen et al. 2007).
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*Methylation*

Methylation takes place on lysine and arginine residues in the histone tails and is controlled by methyltransferases and demethylases. Methylation at lysines may be one of three different forms: mono-, di-, or trimethyl and at arginines mono- or dimethyl (asymmetric or symmetric). In contrast to acetylation and phosphorylation, methylation is a neutral modification and does not alter the charge of the histones (Bannister et al. 2011).

*O-GlcNAcylation*

Recently, evidence has accumulated that O-GlcNAc is also part of the histone code (Sakabe et al. 2010). After this was discovered, several other studies followed and confirmed the presence of an O-GlcNAc sugar residue on different histone proteins in mammalian organisms (Table 1.3). O-GlcNAcylation occurs on serine and threonine residues and is a highly dynamic process (Bannister et al. 2011). The enzymes involved in O-GlcNAc-cycling O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) are responsible for O-GlcNAc addition and removal, respectively (Hanover et al. 2012). O-GlcNAcylation is an ubiquitous PTM found on many nuclear and cytoplasmic proteins and is involved in stress response, regulation of transcription and protein trafficking/turnover. O-GlcNAc is an essential modification, OGT deletion in mammalian cells causes cell death. In plants, two genes spy and secret agent encode OGTs. The simultaneous mutation of both genes is also lethal (Hart et al. 2007). O-GlcNAc targets the same residues as phosphate and they show a complex interplay. They can compete for a single or proximal site or occupy different sites on a substrate (Zeidan and Hart 2010).
Table 1.3 Overview of the different studies confirming the occurrence of O-GlcNAc residues on different histone proteins in mammalian organisms.

<table>
<thead>
<tr>
<th>O-GlcNAc modified AA</th>
<th>Histone</th>
<th>Animal</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Thr101</td>
<td>H1</td>
<td>Calf</td>
<td>Schouppe et al. 2011</td>
</tr>
<tr>
<td>Thr101</td>
<td>H2A</td>
<td>HeLa</td>
<td>Sakabe et al. 2010</td>
</tr>
<tr>
<td>Ser36</td>
<td>H2B</td>
<td>HeLa</td>
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</tr>
<tr>
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<td></td>
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<tr>
<td>Ser10</td>
<td></td>
<td>HeLa</td>
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<td>Calf</td>
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</tr>
<tr>
<td>Ser47</td>
<td>H4</td>
<td>HeLa</td>
<td>Sakabe et al. 2010</td>
</tr>
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**Ubiquitylation**

Ubiquitin is a polypeptide containing 76 residues which is added to histone lysines through the successive action of the following enzymes, E1-activating, E2-conjugating and E3-ligating enzymes. Despite the relatively large size compared to other PTMs, ubiquitylation is also highly dynamic and is involved in both gene activation and gene silencing (Bannister et al. 2011).

**Other modifications**

Histones can be modified with a variety of other modifications. Sumoylation is similar to ubiquitylation and competes with acetylation for the same lysine side chain. Histones can be mono- or poly-ADP ribosylated on glutamate and arginine residues. Deimination involves the conversion of arginine to a citrulline residu (Bannister et al. 2011).

1.2.4 Effect of histone modifications

Histone modifications can be direct, causing structural changes to the chromatin architecture or work indirectly and recruit different effector proteins to the chromatin. As mentioned before, histone acetylation and phosphorylation reduce the positive charge of the histones and therefore disrupt the interaction between histones and DNA. This will lead to a less compact chromatin structure, making the DNA more accessible for multiprotein complexes like those responsible for transcription (Bannister et al. 2011).
Numerous chromatin-associated proteins have been shown to specifically interact with modified histones via many distinct domains. Methylation is recognized by chromo-like domains of the Royal family (chromo (chromatin-binding), tudor, MBT (malignant brain tumor) and non-related PHD (plant homeodomain)) domains, acetylation is recognized by bromodomains, and phosphorylation is recognized by a domain within 14-3-3 proteins (Kouzarides 2007, Bannister et al. 2011). The recruitment of these nonhistone proteins is correlated with diverse functions. The proteins can tether enzymatic activities onto chromatin, deliver specific enzymes, or prevent docking of other nonhistone proteins onto chromatin (Kouzarides et al. 2007). A well studied example is heterochromatin protein 1 (HP1), which binds tri-methylated H3K9 through its chromodomain. HP1 contains binding sites for other HP1 proteins and through binding of those creates an assembly of condensed chromatin. In addition, HP1 also binds with a H3K9 methyltransferase and a deacetylase, which on their turn create new binding sites for HP1. This results into a spreading of the heterochromatin structure along the chromosome until a boundary element is encountered (Kouzarides et al. 2007, Lodish et al. 2013).

Several DNA-based processes such as transcription, DNA repair and DNA replication are regulated by binding of different effector proteins to specifically modified histone proteins. It is believed that phosphorylation of histones assists in the recognition and receptivity of sites where DNA repair is necessary, whereas acetylation has a role in DNA repair (Kouzarides et al. 2007). How epigenetic marks control transcription will be discussed in more detail in the next section.

1.2.5 Influence of chromatin organization on gene regulation

Gene expression is organized by interactions among DNA-binding proteins and regulatory elements. A distinction is made between cis regulatory elements that are encoded in the same sequence as the target gene and trans regulatory elements that are expressed in different regions. Remodeling of the chromatin structure can furthermore affect the interactions among those regulatory elements or transcription factors without changing the genomic sequence (Ha 2013). Hence, mechanisms that alter chromatin structure play a key role in gene regulation. Different mechanisms responsible for controlling chromatin organization are: cytosine methylation, factors influencing nucleosome positioning and composition and post-translational modifications of histones (Lauria and Rossi 2011).

Cytosine methylation

In plants, cytosine methylation (mC) is prevalent in the symmetric CG and CHG contexts (H = C, A or T) and the asymmetric CHH context. Cytosine methylation is highly present within heterochromatin regions enriched with repetitive DNA sequences and transposons, suppressing their activity and as
such maintaining genome stability. DNA methylation is correlated with repression of gene transcription due to the interaction with methyl binding domain proteins which establish a more condense chromatin organization. However, in Arabidopsis, over one third of the expressed genes contain methylation in the transcribed coding region, while mC at the promoter region is quite low (Zemach et al. 2007, He et al. 2011). In the gene body mC presumably has a function in suppressing aberrant transcription from cryptic promoters inside the gene (Lauria and Rossi 2011).

Nucleosome positioning and composition

The position of the nucleosome is regulated by both ATP-dependent chromatin remodeling complexes and the DNA sequence lying underneath. In Arabidopsis, most active genes display nucleosome depletion at the transcription start site (TSS) (Lauria and Rossi 2011, Ha 2013). In addition, all eukaryotes have histone variants of H2A and H3 which are integrated into chromatin during interphase. Histone variants H2A.Z, H2A.X and CenH3, H3.3 are found in all eukaryotes. These variants have diverse functions, but only H2A.Z is involved in gene regulation. H2A.Z regulates gene expression by assisting in preventing DNA methylation and is enriched near the TSS of the gene. It is a necessary but not sufficient factor for transcription (Deal and Henikoff 2011).

Post-translational modification of histones

Regulation of gene expression in euchromatin relies on the recruitment of different chromatin-modifying enzymes by DNA-bound transcription factors. In general, certain histone modifications are linked with activation of transcription, such as acetylation and phosphorylation, with repression of transcription, such as ubiquitylation and sumoylation, while some modifications are linked with both, such as methylation. Since there is an extensive crosstalk between modifications on the same histone or even between histones, these correlations give a good indication but the context is determining whether transcription will occur (Kouzarides et al. 2007).

Data from genome-wide analyses in Arabidopsis, rice and maize were integrated and resulted in epigenomic marks which are positively or negatively linked with transcription (Figure 1.9) (Lauria and Rossi 2011). H3K4me3, H3K36me2/me3, H3K9ac and H3K27ac are located in highly expressed genes but are differentially distributed. The acetylated marks are situated at the 5’-end of the gene and especially at the TSS, and H3K4me3 peaks at the promoter and the 5’-end of the gene, whereas H3K36me2/me3 accumulates over the transcribed region. Also H3K27me3 is preferentially located within the gene, but has a negative effect on transcription. H3K9me2, a heterochromatin mark found in few Arabidopsis genes, spans the promoter and coding region completely. For the remaining
epigenetic marks it is yet unclear if they are positively correlated with transcription. Some of them are possibly involved in making chromatin perceptive for transcription (Lauria and Rossi 2011).

Figure 1.9 Correlation between histone modifications and gene transcription. The distribution of epigenetic marks is mapped on an arbitrary gene, by considering data from genome-wide approaches in Arabidopsis, rice and maize. +/- indicates no precise association with active transcription (Figure adapted from Lauria and Rossi 2011).

The functional epigenetic landscape is much more complex since a single mark can recruit proteins with either activating or repressing functions and has no unique regulatory status. Therefore, an epigenetic mark has to be observed in combination with the neighboring marks and the genomic context for interpretation of their activity (Berger et al. 2007).

**General model of gene regulation through chromatin modifications**

A model was presented by Lauria and Rossi (2011) that describes the different subsequent events that occur during transcription factor-mediated control of gene expression through chromatin modifications. This model shows the sequence of events leading to transcription activation or repression. In general, activation starts with an environmental or developmental pulse which induces the binding of an activator to a specific DNA sequence located in the linker DNA. The activator can have the intrinsic activity to remodel chromatin or can recruit chromatin remodelers which create an open chromatin state characterized by a low nucleosome density and histone PTMs causing a transcription-competent state, but not transcription itself. This open chromatin state is now accessible for other activators capable of binding DNA motifs localized within the nucleosomal regions. Additional chromatin remodelers are recruited, which promote histone exchange (e.g. H2A
with H2A.Z) and modify histones with PTMs permissive for transcription. Subsequently, the pre-initiation complex can be assembled and transcription is started. After RNA polymerase II has passed, different histone PTMs assist in reorganizing the nucleosome and closing the chromatin to prevent aberrant transcription.

Repression of transcription is also initiated by a pulse which recruits a repressor complex to selected regions. This complex recruits on its turn chromatin remodelers causing CG methylation in the promoter, addition of histone marks associated with transcription repression and recruit complexes capable of mediating a compacted chromatin structure (Berger 2007, Lauria and Rossi 2011).

1.2.6 Stress and epigenetic regulation

Organisms are constantly exposed to different environmental stimuli to which they respond with mechanisms of protection or adaptation. The stress responses of the cell are very complex processes which are tightly regulated. A model of a possible stress response is outlined in figure 1.10. A sensor protein fulfills the first step in the response and passes on the message that a stress is present and the need to adapt. The sensor is transported into the nucleus to affect the transcription of a variety of genes. In addition, RNA stability and protein translation rate also determine the stress response. The produced response proteins allow the cell to adapt or protect itself to the changing environment (Smith and Workman 2012).

Many responses to stress are partially controlled by altered transcription of genes which is coordinated by modulating the structure and the accessibility of the genome. Gene expression triggered by a developmental signal or stress is often mediated by modification of histone proteins and sometimes DNA methylation (Chinnusamy and Zhu 2009). For example, heat shock and oxidative stresses can loosen the packing of histones around the DNA. Furthermore, a number of stresses can alter the localization or activity of chromatin modifying enzymes and thus influence the distribution of PTMs on histone proteins (Smith et al. 2012). Interestingly, specific chromatin modifications and proteins do not necessarily have the same function under steady state conditions compared with stress situations. H3K4 methylation is generally known as an activator of transcription but upon stress induction in yeast this epigenetic mark was correlated with repression of genes involved in ribosomal protein production and RNA maturation (Weiner et al. 2012). Stress-induced modifications are mostly reset to a basal level once the stress is removed, although some modifications are stable and can be inherited across mitotic or even meiotic cell divisions (Chinnusamy and Zhu 2009).
Figure 1.10 Cellular responses to stress and environmental factors. Stresses are sensed by factors located inside or outside the cell. This triggers the translocation of the sensor to the nucleus and increases the transcription of specific genes. Finally, response proteins are translated and help the cell to adapt to the new situation. In higher eukaryotes, cells may send signals to neighboring cells to assist in a larger stress response encompassing many cells and tissues. Figure adapted from Smith and Workman 2012.

**Abiotic stress**

The regulation of stress-responsive genes is often mediated by chromatin remodeling. For example, in response to drought stress, the coding regions of drought-responsive Arabidopsis genes are enriched with acetylation of H3K9 and trimethylation of H3K4, which correlates with gene activation (Kim et al. 2008). Sokol et al. (2007) used tobacco BY-2 cells and A. thaliana T87 cell lines to study the nucleosomal response to high salinity and cold stress. The response is characterized by a transient peak of H3S10 phosphorylation, immediately followed by acetylation of lysine 14 of histone H3 and acetylation of H4. For both stresses this response was correlated with the induction of specific stress-responsive genes (Sokol et al. 2007).

**Development under stress**

Plants avoid the effects of stress during reproduction and other critical growth phases through chromatin remodeling. In Arabidopsis, flowering is repressed by the *flowering locus (flc)* gene which directs the transition from vegetative growth to flowering. Gene expression of *flc* is regulated by histone variants and PTMs. During vegetative growth transcription of *flc* is activated by several
histone modifications, H3K4me3, H3K36, acetylation of H3, ubiquitination of H2B, and the incorporation of histone variant H2A.Z. When developmental and environmental conditions are optimal, \textit{flc} is silenced by deacetylation of H3, demetylation of H3K4 and methylation of H3K27, H3K9 and arginine residues in H3 and H4, which leads to silencing of the gene and flowering of the plant (Chinnusamy and Zhu 2009, Deal and Henikoff 2011).

\textit{Stress caused by pathogen attack}

Upon perception of pathogens, the plant defense response is activated by remodeling of the chromatin configuration. Hereby, various histone-modifying enzymes and chromatin remodelers are expressed or modified leading to a unique histone code and subsequent transcription of defense-related genes. However, pathogens developed mechanisms to subvert innate immunity based on the conserved nature of chromatin-remodeling complexes. For example, infection of Arabidopsis by \textit{Agrobacterium tumefaciens} causes plant diseases by transferring a DNA segment (T-DNA) into the plant genome and uses the chromatin remodeling machinery of the plant cell to accomplish this T-DNA integration (Ma et al. 2011).

Besides histone modifications, DNA methylation is also correlated with infection by pathogens. Changes in DNA methylation are stable, required for stress protection and are frequently transmitted to the following generations (Boyko and Kovalchuk 2008). TMV induces an increase in the somatic and meiotic recombination rates in tobacco because of DNA hypomethylation. Furthermore, the progeny of infected plants also showed increased levels of global genome methylation and hypomethylation in the LRR-containing loci of the TMV (\textit{N}-gene) resistance gene. As a consequence, the TMV resistance gene is rearranged more frequently which favours stress adaptation (Boyko \textit{et al.} 2007).
Chapter 2

Jasmonate response of the *Nicotiana tabacum* agglutinin promoter in *Arabidopsis thaliana* and *Nicotiana tabacum*
Chapter 2: Jasmonate response of the *Nicotiana tabacum* agglutinin promoter in *Arabidopsis thaliana* and *Nicotiana tabacum*

Adapted from


Chapter 2: Jasmonate response of the *Nicotiana tabacum* agglutinin promoter in *Arabidopsis thaliana* and *Nicotiana tabacum*

2.1 Abstract

Nictaba is a carbohydrate-binding protein (also called lectin) that is expressed in several Nicotiana species after treatment with jasmonates and insect herbivory. Analyses with tobacco lines overexpressing the *Nictaba* gene as well as lines with reduced lectin expression have shown the entomotoxic effect of Nictaba against Lepidopteran larvae, suggesting a role of the lectin in plant defense. Until now, little is known with respect to the upstream regulatory mechanisms that are controlling the expression of inducible plant lectins. Using *A. thaliana* and *N. tabacum* plants stably expressing a promoter-β-glucuronidase (GUS) fusion construct, it was shown that jasmonate treatment influenced the *Nictaba* promoter activity. Histochemical and fluorometric techniques were used to follow Nictaba promoter activity during the development. In Arabidopsis, a strong GUS staining pattern was detected in very young tissues (the apical and root meristems, the cotyledons and the first true leaves), the promoter activity decreased when plants were getting older. In tobacco plants, GUS staining was predominantly detected in the cotyledons, the leaves and the roots during the youngest plant stages. As the plants grow older GUS staining was mostly present in the older leaves. A detailed comparative analysis was made of the GUS staining results obtained in transgenic Arabidopsis and tobacco lines. Overall, the expression patterns in Arabidopsis and tobacco are similar but still some differences were found especially in the very young tissues.

2.2 Introduction

Jasmonic acid (JA), its precursors and derivatives, collectively called jasmonates, constitute a family of bioactive oxylipins that regulate many physiological plant responses to environmental and developmental signals. As genuine plant hormones they affect a variety of plant processes including defense against insects and pathogens, abiotic stresses, growth, development, fertility and senescence (Wasternack 2007, Pauwels et al. 2008). However, the outcome of jasmonate signaling is not the result of the action of a single molecule but rather reflects part of an integrated signaling network comprising many other molecules such as ethylene, salicylic acid and abscisic acid (Lorenzo et al. 2005).

It is well documented that external stimuli such as wounding, pests, pathogens or developmental cues can elicit jasmonate synthesis from the fatty acid α-linolenic acid through the octadecanoid pathway (Wasternack 2007). After release from the plastid membrane into the stroma of the chloroplasts, α-linolenic acid is converted by several enzymatic reactions into the jasmonate-precursor 12-oxophytodienoic acid, better known as OPDA. Afterwards, OPDA is transported to the peroxisomes where it becomes reduced and converted via the fatty acid β-oxidation reactions to form JA. Finally, the biologically active compound JA can act by itself, or can be metabolized into a
whole array of related jasmonates that can have unique signaling properties different from JA (Mithöfter et al. 2005, Wasternack 2007).

The activation of jasmonate responses occurs by a reprogramming of transcription through the action of several jasmonate activators, such as the MYC2 transcription factor (Boter et al. 2004, Lorenzo et al. 2004). Without any jasmonate signal, MYC2 action is repressed by certain members of the JAZ protein family (Chini et al. 2007, Thines et al. 2007). During jasmonate signaling, JA is converted to its Ile conjugate (Lorenzo et al. 2004) which binds to the F-box protein COI1, thereby increasing interaction of COI1 with JAZ proteins which leads to assembly with the Skp1-like proteins ASK1, ASK2 and CULLIN-1 into a functional SCF<sub>COI1</sub> complex, an E3 ubiquitin ligase. Consequently, the JAZ proteins are labeled for degradation via the ubiquitin / 26S proteasome pathway. As such, MYC2 repression is released, resulting in the expression of jasmonate-inducible genes. To date, MYC2 was shown to activate numerous JA-dependent processes (McGrath et al. 2005, Kazan et al. 2008).

Recently, Memelink (2009) summarized the promoter sequences and transcription factors known to be involved in jasmonate-responsive gene expression. The most common jasmonate-responsive promoter elements are the GCC motif and the G-box. The GCC motif was reported to play a role in the jasmonate responsiveness of the <i>pdf1.2</i> promoter. Analysis of the promoters of jasmonate-responsive Arabidopsis genes showed that G-box (CACGTG) or G-box-like sequences (e.g. AACGTG) are over-represented promoter elements. In addition, TGACG sequences and the jasmonate-responsive elements JASE1 and JASE2 were also identified as jasmonate-responsive promoter elements (Memelink 2009, Jensen et al. 1999).

The expression profile of numerous genes is known to be upregulated after jasmonate signaling. One of these jasmonate-inducible proteins is the tobacco lectin Nictaba. Plant lectins are proteins that contain at least one non-catalytic domain that can reversibly bind to carbohydrate structures (Van Damme et al. 2008). Nictaba or <i>N. tabacum</i> agglutinin is a nucleocytoplasmic lectin of 39 kDa that is expressed in tobacco plants (Chen et al. 2002). The protein typically recognizes glycoproteins containing high-mannose or complex N-glycans and interacts with numerous nuclear and cytosolic glycoproteins (Lannoo et al. 2006a). Similar to other nucleocytoplasmic lectins, Nictaba expression is induced by (a)biotic stress factors (Lannoo and Van Damme 2010). Under normal environmental conditions, Nictaba is not detectable in tobacco plants but the lectin is specifically expressed after treatment with JA or some of its derivatives such as methyl jasmonate (JAME) as well as after insect herbivory. However, lectin expression is not altered by wounding. Until now no other plant hormones or abiotic and biotic stresses were found to induce Nictaba expression (Lannoo et al. 2007a, Vandenborre et al. 2009). After induction by insect herbivory, Nictaba clearly shows
entomotoxic effects on Lepidopteran larvae, indicating a role in plant defense (Vandenborre et al. 2010).

To understand how the jasmonate-induced Nictaba expression is regulated, we identified and analysed the promoter region controlling a Nictaba gene from N. tabacum cv Samsun NN plants. Therefore the Nictaba promoter region was fused to the gus gene to investigate its activity and jasmonate-dependent tissue-specific expression during development in transgenic Arabidopsis and tobacco plants. The data presented here show a strong Nictaba promoter activity especially in younger tissues after JAME treatment of the transgenic Arabidopsis plants. We also present the first evidence for a low expression of Nictaba in the roots of tobacco plants after 12 h cold treatment as well as after JAME treatment. In transgenic tobacco plants, histochemical as well as quantitative GUS stainings were performed. The data obtained for expression of the Nictaba promoter-GUS constructs in tobacco were compared in detail to the results obtained in A. thaliana.

2.3 Results

2.3.1 Isolation of Nictaba promoter sequences from N. tabacum cv Samsun NN

Using the GenomeWalker™ DNA kit, five 1 kb fragments were isolated from genomic DNA of N. tabacum cv Samsun NN, referred to as IL12, IL20, NL32, NL37 and NL40. Sequence comparisons revealed that all genomic fragments contained new sequence information in the upstream region of the intron-containing Nictaba sam3 gene (GenBank ID: DQ155403 (Lanno et al. 2006a)). The tetraploid tobacco cultivar N. tabacum cv Samsun NN possesses 4 genes encoding the Nictaba protein: sam1, sam2, sam3 and sam4 (Genbank DQ155401, DQ155402, DQ155403 and EF115364 resp.) (Lanno et al. 2006a, Lanno 2007b). These lectin genes differ from each other in the coding sequence in the absence or presence as well as the length of the intron sequences. Despite the small differences in their exon sequences, transcripts of all these genes are translated into an almost identical Nictaba protein of 165 amino acid residues. To confirm that the isolated sequences really correspond to the promoter sequences of the Nictaba gene, additional PCR amplifications were performed on genomic DNA from tobacco using promoter (forward)- and gene (reverse)-specific primers. All promoter sequences were completed with the full-length downstream coding sequence of Nictaba up to the stopcodon TAA. Multiple sequence alignment of the identified promoter sequences revealed 92% sequence similarity (67% sequence identity) between these sequences (Delporte et al. 2011). A phylogenetic tree groups the identified Nictaba promoter sequences into 3 clusters (Figure 2.1). A major difference between the IL and the NL clusters concerns a stretch of 10 nucleotides 35 nt upstream of the ATG start codon, which is only present in the IL promoters. NL37
Chapter 2: Jasmonate response of the *Nicotiana tabacum* agglutinin promoter in *Arabidopsis thaliana* and *Nicotiana tabacum*

shows the highest sequence homology (95%) with the other 2 NL promoters NL32 and NL40, but is separated from them in the tree since it lacks part (200 nt) of the upstream promoter sequence.

![Phylogenetic tree](image)

**Figure 2.1** Phylogenetic tree of different 5′ upstream regulatory sequences controlling the *Nictaba sam3* gene. The analysis is based on a multiple sequence alignment of all promoter sequences made in ClustalW (Delporte *et al.* 2011). Numbers correspond to bootstrap values of 1000 iterations.

### 2.3.2 In silico analyses of the *Nictaba* promoter sequence

An extensive search was performed on the *Nictaba* promoter sequences IL12 and NL32 promoters (representing the outer branches of the phylogenetic tree) for the presence and the location of known cis-acting regulatory DNA elements (Figure 2.1). Since both promoter sequences yielded very similar results, only the analysis for the NL32 promoter is shown in detail (Table 2.1).

Since Nictaba is considered a genuine jasmonate-inducible protein (Lannoo *et al.* 2007a, Vandenborre *et al.* 2009), especially JA-responsive promoter elements were searched in the *Nictaba* promoter sequences. Known JA-responsive promoter elements are the GCC-motif, G-box, TGACG sequences, JASE1 and JASE2 (Memelink 2009). Searches in the Riken Arabidopsis and Genome Encyclopedia (RARGE) for jasmonate-responsive promoter elements retrieved JASE1ATOPR1, which appeared once in the NL32 promoter with 2 mismatches. In addition, there are 3 G-box like sequences present in the *Nictaba* promoter sequences. Using the PLACE plant promoter databases, the JA-responsive element T/GBOXATPIN was identified twice.

Searches using the PLACE and PLANTCARE plant promoter databases also suggested the occurrence of multiple putative cis-acting elements responsive towards non-JA-associated hormone signaling (Table 2.1 and Figure 2.2). Different elements putatively related to abiotic responsive gene expression were also identified in the promoter sequences for NL32. The majority of these elements are involved in light responsiveness, a minor part concerns response towards etiolation and cold treatment (Table 2.1).
### Chapter 2: Jasmonate response of the *Nicotiana tabacum* agglutinin promoter in *Arabidopsis thaliana* and *Nicotiana tabacum*

Table 2.1 Potential *cis*-acting regulatory elements identified in the NL32 promoter using an *in silico* pattern search against the PLACE database.

<table>
<thead>
<tr>
<th>Function</th>
<th>Element</th>
<th>Element core sequence</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response to GA</td>
<td>TATCGAGCA05AMY1</td>
<td>TATCGA</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>GAREAT</td>
<td>TAACAAR</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>WRKY7I0S</td>
<td>TGAC</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>PRYMDINEBOXOSRAMY1A</td>
<td>CCTTTT</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>TATCCACHVAL21</td>
<td>TATCCAC</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>CAREOSREP1</td>
<td>CAACTC</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>GADOWNAT</td>
<td>ACGTGTC</td>
<td>1</td>
</tr>
<tr>
<td>Response to pathogen attack</td>
<td>WBOXATNPR1</td>
<td>TTGAC</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>WBOXOPCHWRKY1</td>
<td>TTTGACY</td>
<td>1</td>
</tr>
<tr>
<td>Response to ABA</td>
<td>MYB1AT</td>
<td>WAACCA</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>ACGBABREMTIFA2OSEM</td>
<td>ACGTGKC</td>
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<td></td>
<td>ABRERATCAL</td>
<td>ACGTG</td>
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<tr>
<td>Response to auxin</td>
<td>ARFAT</td>
<td>TGTCTC</td>
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</tr>
<tr>
<td>Response to JA</td>
<td>T/GBOXATPIN2</td>
<td>ACGTG</td>
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<tr>
<td>Response to cold stress</td>
<td>LTRE1HYBLT49</td>
<td>CCGAAA</td>
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<td></td>
<td>MYBCOREAT</td>
<td>AACGG</td>
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<td>ACGTATERD1</td>
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<tr>
<td>Response to light</td>
<td>SORL1PA1T</td>
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<td>GT1GMSCAM4</td>
<td>GAAAAA</td>
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<td>CIACADIANLHIC</td>
<td>CAANNNNATC</td>
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<td>GATA8BOX</td>
<td>GATA</td>
<td>12</td>
</tr>
<tr>
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<td>PRECONSCRHSP70A</td>
<td>SGGAYRRNNNNNNNNNNNNNND</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>TBOXAT7GAPB</td>
<td>ACTTTG</td>
<td>1</td>
</tr>
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</table>
Figure 2.2 Promoter sequence of NL32. Putative cold- and JA-responsive cis-acting regulatory elements and typical promoter elements (CAAT and TATA boxes) are indicated on the sequence. JA-responsive elements are: JASE1ATOPR1, G-box like sequence, T/GBOXATPIN; Cold-responsive elements are: LTRE1HVBLT49, MYBCOREAT, MYBST.
2.3.3 Histochemical analyses of GUS activity in transgenic Arabidopsis plants

The expression of the Nictaba promoters IL12 and NL32 was examined using transformed A. thaliana ecotype Col-O plants that contained promoter/GUS reporter gene fusion constructs. Both IL12 and NL32 promoter sequences contained approximately 900 bp of 5' upstream sequence of the Nictaba gene sam3 including its 5' untranslated region (Delporte et al. 2011). Although these promoter sequences contain the typical promoter elements, one cannot be sure that these 900 bp fragments represent the full length promoter sequence. Nevertheless, these fragments were sufficient to drive GUS expression. Promoter activities were examined in different plant tissues during the development of Arabidopsis, treated with 50 µM JAME or with water for 24 hours. Six different plant stages were analyzed starting with 1) 3-day-old seedlings, 2) 9-day-old seedlings, 3) adult plants with closed flowers, 4) adult plants with open flowers, 5) adult plants with green siliques and finally 6) adult plants with brown siliques. Since similar patterns of expression were detected for both promoter constructs throughout development only the results for NL32 will be detailed below. Arabidopsis lines in which the cauliflower mosaic virus 35S promoter was used to drive recombinant GUS expression were used as controls.

2.3.3.1 Plant stage 1: 3-day-old seedlings

Similar to the cauliflower mosaic virus (CaMV) 35S-GUS control construct (Results not shown) the JAME-treated seedlings with the NL32-GUS construct showed a GUS positive staining of the cotyledons preferentially around the vascular tissue and at the tips of the cotyledons (Figure 2.3 a). The petioles showed a strong GUS staining, mainly around the vascular tissue. More than half of the stomatal cells showed intense staining, which was always visible in both guard cells (Figure 2.3 a-1). A very intense GUS activity was also observed in the shoot apex, especially at the base of the trichomes. In the hypocotyl, GUS expression was confined to the vasculature and continued in the central cylinder of the root. Interestingly, GUS activity was high near the root tip (Figure 2.3 a-2).

In water-treated seedlings (Figure 2.3 b) GUS expression was confined to the major veins of the cotyledons. The vascular tissue from the roots and the shoot apex showed a much lower GUS activity. In contrast to JAME-treated seedlings, no GUS expression was observed in the root tip of water-treated seedlings (Figure 2.3 b-1).
Figure 2.3 Histochemical analysis of GUS activity in Arabidopsis seedlings directed by the NL32 promoter. GUS expression was analyzed for 3-day-old (a-b) and 9-day-old seedlings (c-d) after 24 h treatment with JAME (a and c) or water (b and d). Details of the stomata (a-1) and root tip (a-2; b-1) are shown.

To confirm this first series of results, cross sections of cotyledons from 3-day-old seedlings were made (Figure 2.4). Similar to the previous results, the JAME-treated sections showed an intense GUS staining, especially around the vascular tissue (Figure 2.4 a-1) whereas GUS staining in water-treated seedlings was only confined to the vascular tissue (Figure 2.4 b-1).
Figure 2.4 Cross sections of cotyledons from GUS stained Arabidopsis seedlings transformed with NL32/GUS promoter construct. GUS expression was analyzed in 3-day-old seedlings after 24 h treatment with JAME (a) or water (b). Details of the vascular tissue (vt) and surrounding mesophyll cells (m) are shown (a-1, b-1).

2.3.3.2 Plant stage 2: 9-day-old seedlings

In 9-day-old seedlings having 2 fully matured cotyledons and the first 2 true leaves, the pattern of the GUS histochemical staining had changed. GUS staining of the mature cotyledons treated with JAME was typically seen only in the veins (Figure 2.3 c), similar to the expression pattern seen in water-treated plants (Figure 2.3 d). However, JAME-treated seedlings showed a very strong GUS staining of the first true leaves (Figure 2.3 c), with clear staining of the trichomes on the young leaves, especially near the base. GUS activity was also observed in the vasculature of the hypocotyl, the root and in the apex of initiating lateral roots (Figure 2.3 c). Compared to JAME-treated plants the staining of the vasculature in the hypocotyl and the primary and lateral roots of water-treated plants was much weaker (Figure 2.3 d). In Arabidopsis lines expressing the CaMV35S-GUS construct, a strong GUS staining was observed in all plant tissues, except for the hypocotyl (Results not shown).
2.3.3.3 Plant stages 3-6: plants older than 1 month

In 1-month-old Arabidopsis plants, the GUS staining was observed in all organs (Figure 2.5). Clearly stronger GUS staining was present in developing leaves than in older mature leaves where GUS staining was typically seen in the veins. GUS expression was detected diffusely in the leaves. In rosette leaves of JAME-treated plants (Figure 2.5 a-5), GUS expression was visible in all veins except for the middle vein, with a stronger expression at the edges of the leaf where mesophyll cells show very diffuse GUS staining. Cauline leaves showed a similar but more intense colouring pattern with strong staining of the trichomes at the base of the leaf (Figure 2.5 a-6). In water-treated plants a very weak GUS expression was observed in rosette and cauline leaves (Results not shown). The staining was confined to veins located at the edges of the leaf, mainly near the base of the leaf.

Floral organs showed GUS expression in the veins of the sepals, the style, the stigma and the ovary (Figure 2.5 a-3 and 2.5 a-4). Embryos of different age present in the seeds did not show any GUS staining (Results not shown). Some GUS expression was associated with the funiculus that attaches the developing seed to the siliques, as well as in the septum of the siliques (Figure 2.5 a-1 and 2.5 a-2). However, very similar results were obtained for water-treated and JAME-treated plants. But only flowers derived from JAME-treated plants also showed GUS expression in the filament of the stamen.
Figure 2.5 Histochemical analysis of GUS activity in adult Arabidopsis plants directed by the NL32 promoter. GUS expression was analyzed for 1-month-old plants after 24 h JAME (a and 1, 3, 5, 6, 7) or water treatment (2, 4, 8). Details of the siliques (1-2), flowers (3-4), rosette (5) and cauline leaves (6) and roots (7-8) are shown. A magnification is shown of the trichomes at the base of the cauline leaf (6a) and of the roots (7a).

In roots, a very high GUS expression was seen especially in the vasculature of the primary root, whereas lateral roots showed a weaker GUS expression as more lateral roots are formed (Figure 2.5 a-7 and 3a-8). Plants harbouring the CaMV35S-GUS control construct showed a complete blue staining of rosette and cauline leaves, flowers, siliques and roots (Results not shown).
2.3.4 Nictaba expression in the roots of *N. tabacum* cv Samsun NN plants

The histochemical GUS staining experiments in Arabidopsis demonstrated a clear activity of the *Nictaba* promoter in the roots of the transformed plants. However, until now, Nictaba activity was never reported in tobacco roots (Chen *et al.* 2002, Lannoo *et al.* 2007a). Therefore the root-specific expression of Nictaba was studied after JAME-treatment of the tobacco plants (*N. tabacum* cv Samsun NN) using agglutination assays, Western blot analysis and ELISA (Figure 2.6). Since Nictaba is primarily expressed in JAME-treated tobacco leaves, this plant material was used as the positive control. Using an agglutination assay, Nictaba activity was only detected in protein extracts from leaves originating from JAME-treated plants, whereas the extracts of the roots of the same plants did not contain detectable amounts of lectin activity. Extracts from leaves or roots in the control treatment did not show any lectin expression.

![Figure 2.6](image)

**Figure 2.6** Quantification of Nictaba levels after JAME and cold treatment. Nictaba increase in leaves and roots of tobacco was measured using an ELISA (A). Analyses were done with three-week old tobacco plants treated with JAME for 24 h or eleven-week old plants subjected to a cold stress for 12 h. Data represent the increase in lectin concentration compared to the control (no treatment). Data represented are the mean (± SE) of three independent biological repeats. Asterisks indicate significant differences compared to untreated roots or leaves (t-test; * p < 0.05; ** p < 0.001; *** p < 0.01). Root (R) and leaf (L) extracts from tissues treated with different concentrations of JAME were also subjected to Western blot analysis (B).

However, the combination of SDS-PAGE and Western blot analysis using an antibody specifically directed against Nictaba allowed to visualize a clear protein band for Nictaba in protein extracts from both tobacco leaves and roots sampled from JAME-treated plants (Fig 2.6 B). This result was confirmed and quantified by a more sensitive ELISA assay. As shown in Figure 2.6, Nictaba concentrations increased in leaves and roots after JAME-treatment, confirming a low Nictaba...
expression in the roots as observed in the promoter-GUS staining experiments. At higher JAME concentrations, Nictaba expression decreases in leaves. This is in agreement with earlier observations and is probably caused by the senescence of the leaf, resulting in a decrease of the total protein content (Lannoo and Van Damme 2010).

In silico analyses of the Nictaba promoter revealed multiple cis-acting elements known to be associated with light and cold stress (Table 2.1). Previously, it was shown that light and dark grown tobacco plants both express equal amounts of Nictaba after JAME treatment (Lannoo et al. 2007a). To investigate the impact of cold treatment on Nictaba gene expression in tobacco, plants were subjected to a cold treatment for 12h and analyzed for lectin activity both in leaf and root extracts. Both Western blot analysis and ELISA confirmed Nictaba expression in the roots after cold treatment. Surprisingly, cold treatment did not affect lectin accumulation in the leaves (Figure 2.6).

2.3.5 Histochemical GUS staining after JAME treatment in tobacco

The same Nictaba promoter constructs IL12 and NL32 were used to transform tobacco. Since Nictaba is considered a genuine jasmonate-inducible lectin, transgenic tobacco plants expressing the promoter-GUS constructs were treated with JAME and promoter activity was analyzed using histochemical stainings. Therefore, five independent transgenic lines of the T1 generation were analyzed for GUS staining for each promoter construct after treatment with JAME. Transgenic tobacco plants treated with water were used as a negative control.

To follow the promoter activity during the life cycle of the tobacco plant, five different plant stages were analyzed, starting with 1) 24h old seedlings, 2) 48h old seedlings, 3) 1-week-old plants, 4) 3-week-old plants, and finally 5) flowering plants. Histochemical analyses were performed on complete plants for plant stages 1 to 4. In case of plant stage 5, only the floral organs were examined. Both promoter constructs yielded very similar GUS expression patterns.

2.3.5.1 Plant stage 1: 24h-old seedlings

In the youngest growth stage, GUS activity was clearly observed after treatment of the seedlings with JAME for 24 hours (Figure 2.7), whereas water treated plantlets did not show any blue staining. Staining was observed in the mesophyll cells as well as in the vascular tissues of 24 h cotyledons whereas the hypocotyl and the apical meristem were clearly devoid of GUS activity (Figure 2.7-a, 2.7-b). Some closed stomatal guard cells also showed a strong GUS staining (Figure 2.7-c). The primary root showed only a faint blue staining in the vascular tissue of the lower part of the root.
corresponding to part of the zone of cell differentiation (Figure 2.7-d). In contrast, there was no expression whatsoever in the secondary roots and in the root tips (Figure 2.7-e).

**Figure 2.7** Histochemical analyses of GUS activity directed by the NL32 promoter in 1-day-old tobacco seedlings treated with JAME. The inset shows the water-treated control plant. Details of the hypocotyl (a), apical meristem (b), stomata (c), root (d) and root tip (e) are shown. The IL12 promoter constructs yielded very similar results.

### 2.3.5.2 Plant stage 2: 48h-old seedlings

Two-day-old plantlets showed a very similar expression pattern as seen in 24h-old seedlings, in that the GUS staining was confined to the cotyledons, particularly to the vascular tissue and some stomatal cells (data not shown). Some GUS staining was also detected in the vascular tissue of the lower part of the root, but not in secondary roots or root tips. However, the GUS intensity is reduced compared to plant stage 1. No promoter GUS activity was detected in the water-treated plantlets.
2.3.5.3 Plant stage 3: 1-week-old plantlets

In 1-week-old plantlets grown *in vitro*, the GUS staining in the cotyledons had almost completely disappeared, and only a weak signal especially at the tip of the cotyledons was still visible. Remarkably, the first leaves showed very strong promoter activity (Figure 2.8). A clear GUS staining was observed in the veins of the leaf and in some stomatal guard cells (Figure 2.8-a). No GUS staining appeared in the apical meristem and the hypocotyl. At this stage a strong blue color was seen in the vascular tissue of the primary, but also of the secondary roots. The blue color is confined to the lower part of the root excluding the root tip (Figure 2.8-b, 2.8-c). No GUS expression was observed in the water treated plantlets.

![Figure 2.8](image)

*Figure 2.8* Histochemical analyses of GUS activity directed by the NL32 promoter in 1-week-old tobacco seedlings treated with JAME. The inset shows the water treated control plant. Details of the stomata (a), root (b), root tip (c) are shown. The IL12 promoter constructs yielded very similar results.
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2.3.5.4 Plant stage 4: 3-week-old plants

An intensive GUS staining was observed in the senescing cotyledons and first true leaves of 3-week-old plants grown in soil (Figure 2.9-a). This GUS staining was especially observed in the vascular tissue and at the outer edges of the leaf. A very similar GUS staining pattern was also visible in the other leaves, although the intensity of GUS staining was lower. Remarkably, the hypocotyl also showed strong GUS expression at this stage of development, especially in the vascular tissue. A strong GUS staining was observed in the vascular tissue of some roots. Particularly the oldest roots showed strong promoter activity, but not in the root tip. The water treated plants did not display any GUS activity (Figure 2.9-b).

![Figure 2.9](image)

**Figure 2.9** Histochemical analyses of GUS activity directed by the NL32 promoter in 3-week-old tobacco seedlings treated with JAME (a) or water (b). GUS staining is visible in the hypocotyl (hy), first true leaves (ftl) and part of the primary root (rt). The IL12 promoter constructs yielded very similar results.

2.3.5.5 Plant stage 5: Flowering plants

Flowers of transgenic plants were subjected to a GUS histochemical assay after treatment with JAME. As shown in Figure 2.10 a-1, the outer rim of the sepals showed a clear blue staining, especially for the IL12 promoter construct. No GUS staining was observed in the style, the stigma and the ovary. Although the NL32 construct showed a similar expression pattern, the GUS staining was much weaker (Figure 2.10 a-2). Water treated flowers did not show any GUS staining (Figure 2.10 b).
2.3.6 Fluorometric GUS activity in tobacco

Since the histochemical analysis does not permit the detection of low levels of GUS, nor measuring an increase in the level of expression, a quantitative GUS assay was also performed on 5-week-old tobacco plants, which had formed at least six leaves. Five fully-expanded leaves, as well as the youngest leaf (including the apical meristem) and the roots were analyzed for GUS expression (Figure 2.11). The cotyledons and the first true leaves of 5-week-old plants were excluded from the test because they were decaying.

As shown in figure 2.11 the highest level of GUS activity for the NL32 promoter was detected in the sixth or in this case the oldest leaf of JAME treated plants. This GUS activity gradually decreased toward the younger leaves but was somewhat higher in the youngest fully expanded leaf (leaf 2). Since the water-treated leaves showed very little GUS activity, there is a significant difference between JAME treated and untreated leaves. However, in the youngest leaf sample (1), no significant difference in promoter activity was detected for water and JAME treated tissues. GUS activity in the roots was also low.

Similar results were obtained for the IL12 promoter (data not shown), with the highest promoter activity in the oldest leaves. In this case the youngest leaves also showed pronounced promoter activity. Quantitative GUS activity measurements for the NL32 promoter yielded values up to 4-fold higher compared to the IL12 promoter.
Figure 2.11 Fluorometric analyses of GUS activity in transgenic tobacco plants harboring the NL32::GUS promoter construct. Plants were subjected to JAME or water treatment. Individual leaves (1-6) and roots (7) were sampled as indicated on the schematic drawing for the tobacco plant. Asterisks indicate significant differences compared to water-treated leaves or roots (t-test; *p < 0.05; **p < 0.01, ***p < 0.005, **** p < 0.001).

2.4 Discussion

2.4.1 Presence of jasmonate responsive elements in the Nictaba promoter sequence

Since Nictaba is a genuine jasmonate-inducible lectin, promoter elements responsible for this jasmonate-responsive character were searched. By checking the promoter sequence for known JA-responsive promoter elements, three G-box like sequence were identified. Plant promoter database searches also identified two JA-responsive elements, JASE1ATOPR1 once and T/GBOXATPIN twice, in the promoter sequence. JASE1ATOPR1 is a JAME-responsive element found in the promoter of the A. thaliana 12-oxo-phytodienoic acid-10,11-reductase (OPR1) gene (He et al. 2001). T/GBOXATPIN is present in the promoter sequences of the tomato defense proteins LAP and PIN2 and is recognized by the bHLH-Leu zipper DNA-binding proteins JAMYC2 and JAMYC10 (Boter et al. 2004, Lorenzo et al. 2004). JAMYC2 is an important JA-inducible transcription factor that positively regulates the defense response towards pest insects. All these elements can contribute to the jasmonate-inducible expression of Nictaba and were associated with cis-acting regulatory motifs present in promoter regions of defense-related proteins.
Database searches also indicated the occurrence of different non-JA-responsive promoter elements, however, other hormone-associated elements are unlikely to be active since earlier experiments showed that of all plant hormones tested only JA and its derivatives affected lectin synthesis (Lannoo et al. 2007a). Also putative cis-elements related to abiotic responsive gene expression were identified, but they need to be verified by functional analysis. In this respect, Nictaba resembles ver2, a jacalin-related lectin in winter wheat. The expression of ver2 can be induced by low temperature treatment and it was shown that ver2 plays an important role in the vernalization process (Yong et al. 2003). A detailed analysis of the ver2 promoter region fused to green fluorescent protein allowed the identification of several possible response elements which are known to respond to cold, abscisic acid, JA, gibberelin and light stress signaling, but experiments actually indicated that the ver2 promoter is only responsive towards abscisic acid, JA and light stress (Feng et al. 2009).

2.4.2 Nictaba promoter activity during development of Arabidopsis

Histochemical GUS staining is a well-established method that allows following promoter activity during plant development. It was clear that the expression of the gus gene and thus the activity of the Nictaba promoter diminished as the plants were getting older (Figures 2.3 and 2.5). This decrease in activity was very obvious when comparing the GUS staining of the cotyledons in plant developmental stages 1 and 2. Whereas the cotyledons and the first true leaves initially showed a very intense staining, the GUS activity dropped as the leaf material was getting older. Similarly in plant stages 3 and 4 GUS staining was much weaker and more diffuse as leaves were expanding.

2.4.3 Nictaba expression in the roots of tobacco plants

Remarkably, the histochemical staining not only revealed Nictaba promoter activity in leaves, but also in roots of Arabidopsis plants, especially in the vascular tissues. This vasculature-related expression contrasts with earlier immunohistochemical assays that have been performed for Nictaba in tobacco plants, where Nictaba expression was only detected in parenchyma cells but not in the phloem tissue (Chen et al. 2002). The vasculature-specific staining of the Nictaba promoter/GUS constructs differs from that of AtPP2-A1/GUS constructs reported by Dinant et al. (2003). AtPP2-A1 is a phloem-specific Arabidopsis lectin that shares a C-terminal domain highly homologous to Nictaba and an unrelated long N-terminal domain which is implied in phloem localisation (Dinant et al. 2003, Beneteau et al. 2010). However, since we cannot exclude the context-dependence of transcriptional regulation, it is possible that the Nictaba promoter sequences react differently in Arabidopsis compared to tobacco plants. It has already been described that the application of JAME alters the activity of enzymes of the oxylipin pathway to a different extent in both plant species (Avdiushko et
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...*al.* 1995). Therefore, experiments were performed to assess the occurrence of Nictaba in roots of tobacco, which confirmed the findings of the GUS experiment. Nictaba is also expressed at low concentrations in tobacco roots.

### 2.4.4 Nictaba promoter activity during development of tobacco

Nictaba promoter constructs yielded high promoter activity in the cotyledons, especially in the youngest plant stages (24h- and 48h-old seedlings). In older plants, GUS staining is apparent in the true leaves, with more intense GUS staining in older compared to younger leaves, suggesting stronger promoter activity in the first leaves of the plant. The latter results were also confirmed by quantitative analyses in 5-week-old plants. A similar age-dependent expression has also been reported for the rice lectin SalT (Garcia *et al.* 1998). In plant tissues with high levels of cell expansion and/or cell division, SalT was expressed to its highest extent, whereas in older plants expression shifted to the oldest leaves.

In agreement with the GUS analyses in *A. thaliana* clear promoter activity was also detected in tobacco roots. In the youngest plant stages (24h-, 48h- and 1-week-old seedlings) primary tobacco roots show GUS expression in the vasculature of the lower part of the root, but not in the root tip. In 3-week old tobacco plants Nictaba promoter activity was observed in the oldest (primary) roots.

### 2.4.5 Comparative analysis between Nictaba promoter activity in Arabidopsis and tobacco

We analyzed the GUS activity driven by the Nictaba promoter in *A. thaliana*. Since Nictaba is a tobacco protein and the genetic background from *Arabidopsis* and tobacco is quite different, these promoter studies were also performed in tobacco. This experimental approach not only allowed to analyze the promoter activity in its original genetic background, but also enabled the comparative analyses of the data obtained in tobacco and thale cress.

A comparative analysis of the Nictaba promoter activity in Arabidopsis and tobacco revealed that at first sight the overall picture of GUS staining was very similar. At the early stages of development, the GUS staining pattern in the cotyledons and leaves of both plant species showed an intensive staining of the mesophyll cells and some stomatal guard cells. GUS activity in these tissues gradually decreased as the plants got older. In 1-week-old tobacco, the cotyledons were completely devoid of GUS activity whereas a low level of GUS activity always remained in the cotyledons of 9-day-old Arabidopsis plants, especially in the vasculature. Remarkably, water treated controls never showed any GUS activity in tobacco, whereas in Arabidopsis some light blue background staining was always present.
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When zooming into detail, some striking differences were observed. First, the apical meristem of tobacco plantlets is completely devoid of GUS staining, whereas in Arabidopsis the meristem tissue showed a very strong blue colour. Second, the hypocotyl does not show any GUS staining in the young stages of the tobacco development. However, in three-week-old tobacco plants, the hypocotyl showed clear GUS activity. GUS expression in the Arabidopsis hypocotyl was observed only in the vascular tissue during the early stages of development (3-day-old seedlings and 9-day-old seedlings).

Third, the GUS staining pattern in the roots of tobacco and Arabidopsis was completely different. During early stages of tobacco development, GUS activity was detected in the lower part of the zone of cell differentiation, but not in the root tip. In older tobacco plants the staining expanded to the complete zone of cell differentiation, but the GUS staining was clearly confined to the primary roots. In contrast, in Arabidopsis, promoter activity was observed in the root system at all stages of development, but GUS expression in the root tip was only observed in the 3-day-old Arabidopsis seedlings. Finally, Nictaba promoter activity was different in the flowers. In tobacco, GUS activity was clearly present at the rim of the petals. However, in Arabidopsis flowers promoter activity was much more pronounced, since GUS staining was detected in the veins of the sepals, but also in the style, the stigma and the ovary.

Transgenic *A. thaliana* and tobacco are frequently used to gain more insight in protein expression of proteins originating from harder-to-transform plant species like rice, soybean and barley. Although, in most reports GUS promoter activity is only analyzed in one species, a few reports have attempted to analyze GUS activity in multiple plant species. In most cases the data reported are quite similar for different species or compared with the original plant species from which the promoter sequence was analyzed, especially when the genes under studies are well conserved among plant species (Stenzel *et al.* 2008, An *et al.* 2009, Singer *et al.* 2011). However, careful analysis of the available literature did reveal some more reports documenting differences in GUS staining patterns obtained for the same promoter when analyzed in a different plant background. Inaba *et al.* (2007) analyzed the expression of the tobacco ASA2 promoter in soybean. Although the GUS staining patterns for tobacco and soybean were quite similar no ASA2 driven expression in seed or pollen was observed in tobacco. Similarly differences in GUS staining were observed when the rice YY2 promoter was transformed in Arabidopsis. In contrast to rice where the GUS staining was confined to the tapetum the complete anthers showed GUS activity in Arabidopsis (Kuriakose *et al.* 2009, Khurana *et al.* 2013). Kobayashi *et al.* (2007) also observed clear differences when GUS analyses were performed for the barley IDS3 promoter in transgenic Arabidopsis and tobacco. Expression occurred mainly in the epidermis of Arabidopsis roots, whereas expression was dominant in the pericycle, endodermis and cortex of tobacco roots.
In the latter two cases, the differences in promoter activity could be attributed to the fact that a promoter from a monocotyledonous species was analyzed in a dicotyledonous background. However, this reasoning does not hold true for our data describing differences in promoter activity observed between tobacco and Arabidopsis. The most striking differences in GUS staining were observed in the apical meristem, the roots and the flowers. Although these differences in GUS expression patterns might look trivial, they could be quite important, taking into account the role of Nictaba in plants cell signaling and defense.

### 2.5 Conclusions

The data presented provide information related to Nictaba expression during the life cycle of the plant and will facilitate the search for potential Nictaba interacting proteins and hence related function during plant development and plant defense. Several very homologous Nictaba promoter sequences controlling gene expression of the Nictaba sam3 gene present in Nicotiana tabacum cv Samsun NN plants were isolated and characterized. The responsiveness of the Nictaba promoter towards jasmonates was clearly observed in JAME-treated seedlings and adult Arabidopsis and tobacco plants. The higher Nictaba promoter activity and thus a higher level of Nictaba in younger tissues can be important for the defensive role of Nictaba against pest insects which preferably consume younger plant tissues. This study contributes to the knowledge about the jasmonate-induced expression of the tobacco lectin Nictaba with regard to its regulation at the transcriptional level.

### 2.6 Materials and methods

#### 2.6.1 Plant material

Seeds of *N. tabacum* cv Samsun NN and *A. thaliana* ecotype Columbia (Col-O) were purchased from Lehle Seeds (Round Rock, Texas, USA). Prior to use, dry seeds were surface sterilized with 70% (v/v) ethanol for 2 min and then with 7% (v/v) NaOCl for 10 min. The seeds were rinsed thoroughly with sterile distilled water before they were sown. To establish an *in vitro* culture, seeds were germinated on solid Murashige and Skoog medium (containing 4.3 g/L MS micro and macro nutrients containing vitamins (Duchefa, Haarlem, The Netherlands), 30 g/L sucrose, pH 5.7 (adjusted with 0.5 M NaOH) and 8 g/L plant agar (Duchefa)). Arabidopsis seeds were imbibed for 2 days at 4°C in the dark before they were transferred to a growth chamber at 21°C with a 16/8 h light/dark photoperiod. Tobacco seeds were incubated directly in a growth chamber at 25°C with a 16/8 h light/dark photoperiod. After germination, Arabidopsis plantlets were transferred to artificial soil (Jiffy-7, columnar diameter
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44 mm, AS Jiffy Products, Drobak, Norway) to grow adult plants. Plant developmental stages 1 and 2 were grown *in vitro*, whereas plant stages 3-6 were grown in artificial soil.

### 2.6.2 Treatment of *Arabidopsis* and tobacco plants

*Arabidopsis* seedlings and plants were floated for 24 h on a 50 µM JAME-solution (dissolved in water). Three biological repeats were used to perform the GUS staining. Control plants were treated similarly with water, here 2 biological repeats were used.

Tobacco seedlings were treated identically. Five biological repeats were performed for the GUS staining of JAME-treated plants. Control plants were treated with water. In the latter case 3 biological repeats were used.

To check whether Nictaba was expressed in the roots, the following setup was used. Three-week-old tobacco plants were treated with 50 µM and 100 µM JAME for 4 days. Therefore, plants were removed from the soil, all soil was removed from the roots, and the roots were incubated in jars containing the JAME solution. Control plants were treated similarly with water. Tobacco leaves and roots from three biological repeats were analyzed for lectin activity. Leaves of comparable age and position on the plant were used for all assays.

Eleven-week-old tobacco plants, grown in pot soil, were subjected to cold stress by incubating the plants at 4°C for 12 h. Control plants were kept in the growth chamber at 25°C. Leaves of comparable age and position on the plant were used for all assays.

### 2.6.3 Amplification of the Nictaba promoter from tobacco

Genomic DNA was isolated from leaves of tobacco plants as described before (Lannoo *et al.* 2007a). The 5’ upstream regions of the *Nictaba sam3* gene were isolated using the Universal GenomeWalker™ Kit (Clontech Laboratories, Inc, CA, USA). Four GenomeWalker libraries were constructed according to the manufacturer’s instructions. After phenol/chloroform purification, libraries were used as templates in a nested PCR reaction in a GeneAmp PCR System 9600 Apparatus (PerkinElmer Corp., Norwalk, CA, USA) using the BD Advantage™ PCR Enzyme system of Clontech. A first PCR was performed using the AP1 Genome Walker primer (Clontech) and the gene-specific primer evd 89 using cycling parameters: 2 min 94°C, 7x (2 sec 94°C, 3 min 70°C), 32x (2 sec 94°C, 3 min 65°C), 4 min 65°C. PCR products were 1:10 diluted and used as templates in a subsequent PCR with the AP2 Genome Walker primer and the gene-specific primer evd 88. PCR cycling parameters were identical as for the first PCR. To complete the obtained promoter sequences with the 3’ downstream coding sequence of the *Nictaba* gene, nested PCR was performed using the promoter-
specific primer evd 155 and the 3’UTR-specific primer evd 43 in a first amplification round, and primer set evd 155 and evd 66, specific for the 3’ end of exon 3 of the Nictaba gene, in a second PCR amplification reaction. All PCR primers used are shown in Appendix A. All secondary PCR products were ligated into the pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen, California, USA) and transformed into E. coli Top10 cells using heat shock transformation. Transformants were selected by colony PCR. Plasmid DNA inserts were sequenced with the universal M13 forward and reverse primers at the VIB Genetic Service Facility (Antwerp, Belgium). New sequence data have been deposited in GenBank under accession numbers EF115365, EF115366, EF115368, EF115369 and EF115370.

2.6.4 In silico analysis

DNA sequences were analyzed by VectorNTI 10.0 (Invitrogen) and BioEdit 7.0.4.1. Multiple sequence alignments were generated using ClustalW 1.83. The output was visualized using BoxShade 3.21. Based on the ClustalW data, a PAUP phylogenetic tree was constructed using TreeIllustrator (Trooskens et al. 2005). Promoter sequences were scanned for the presence of cis-elements identical or similar to motifs registered in 2 plant cis-acting regulatory elements databases, PlantCARE (Lescot et al. 2002) and PLACE 28.0 (Higo et al. 1999), using the database-associated search tools. Perl scripts were used for computational analyses on all elements found in the two databases.

2.6.5 Construction of promoter-GUS reporter vectors

For the expression of the promoter-GUS constructs, the Nictaba promoter sequences IL12 and NL32 were cloned into the pKGWFS7.0 vector (Karimi et al. 2002) using the Gateway™ Cloning technology of Invitrogen. Therefore the Nictaba promoter sequences were amplified as attB-PCR products in a nested PCR using Taq DNA Polymerase (Invitrogen) and primers evd 108 and evd 109 in a first reaction, and primers evd 2 and evd 4 in a second reaction to complete the attB sequences (Appendix A). Using this PCR strategy, constructs were made of the promoter sequences including the 5’ UTR of the Nictaba sam3 gene directly fused to the ATG codon of the E. coli GUS gene. All attB-PCR products were homogeneously recombined in the pDONR221 vector (Invitrogen) using the Gateway™ BP Clonase™ Enzyme mix (Invitrogen). Entry clones were subsequently transferred into E. coli strain Top10 cells using heat shock transformation. Transformants were selected by colony PCR. Plasmid DNA was sequenced with primers evd 108 and evd 109. Finally, LR reactions were performed with selected entry clones and the pKGWFS7.0 destination vector using the Gateway™ LR Clonase™ Enzyme mix (Invitrogen) to obtain expression clones. The expression clones were transformed via
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heat shock into *E. coli* cells. Transformants were selected on LB agar plates containing spectinomycin (25 µg/ml) and screened with PCR.

2.6.6 Transformation of *A. thaliana* and PCR analysis

The binary vectors containing promoter-GUS fusions were introduced into *Agrobacterium tumefaciens* GV3101 cells using electroporation with a Gene Pulser® (Bio-Rad) with pulse settings 2 kV, 25 µF and 200 Ω. Transformants were selected on YEB agar plates containing spectinomycin (50 µg/ml) and used for transformation of *A. thaliana* plants ecotype Col-O by floral dip. Transformed seedlings were selected on MS agar plates containing kanamycin (50 µg/ml). DNA was purified from 2-week-old seedlings using the FastDNA® SPIN Kit (Qbiogene Inc., Carlsbad, USA). Integration of the T-DNAs into the plant genome was checked by PCR on genomic DNA using the GUS-specific primers GUS-F and GUS-RV and the kanamycin-specific primers evd 463 and evd 261 (Appendix A). Cycling parameters were as follows: 2 min 94°C, 28x (15 sec 94°C, 30 sec 50°C, 1 min 72°C), 5 min 72°C.

2.6.7 Transformation of *N. tabacum* cv Samsun NN and selection of transgenic lines

The binary vectors containing the Nictaba promoter-GUS fusions were introduced into *A. tumefaciens* LBA4404 cells using tri-parental mating. Transformed cells were selected on YEB agar plates (5 g/L beef extract, 5 g/L peptone, 1 g/L yeast extract, 5 g/L sucrose) containing spectinomycin (75 µg/ml) and used for transformation of *N. tabacum* cv Samsun NN leaf disks. Transformed plants were grown until they set seeds. These seeds were selected on MS agar plates containing kanamycin (100 µg/ml). These seedlings from the T$_1$ generation were used for the different experiments.

After shoots and roots emerged from the transformed calli, the integration of the T-DNA into the plant genome was checked by PCR on genomic DNA using the GUS-specific primers evd 596 and evd 597 (Tm = 50°C). Actin-specific primers evd 252 and evd 253 (Tm = 55°C) were used to verify the DNA quality. Cycling parameters were as follows: 2 min 94 °C, 30x (15 s 94 °C, 30 s Tm °C, 1 min 72 °C), 5 min 72 °C. RNA expression after JAME (50 µM) treatment was checked. Therefore RNA was extracted from JAME-treated leaves and transcribed into cDNA. The presence of GUS cDNA was analyzed by PCR using the same conditions as indicated above. cDNA quality was examined using primers evd 282 and evd 283 (Tm = 55°C) to amplify part of the ribosomal protein L25 (Genbank accession number L18908). All sequences for the PCR primers are shown in Appendix A.
Chapter 2: Jasmonate response of the *Nicotiana tabacum* agglutinin promoter in *Arabidopsis thaliana* and *Nicotiana tabacum*

2.6.8 Histochemical and fluorometric analyses of GUS activity

The protocol for histochemical staining of β-glucuronidase (GUS) was adapted from (Jefferson 1987). For each construct, 5 independent transgenic lines of a T3 generation were used. Briefly, *Arabidopsis* seedlings or adult plants from a T3 generation were incubated in 90% acetone for 30 min at 4°C, followed by 3 washes for 5 min in phosphate buffer (0.1 M NaH₂PO₄·H₂O and 0.1 M Na₂HPO₄, pH 7.0). Subsequently, seedlings or adult plants were incubated for 30 min at 37°C in GUS preincubation buffer (phosphate buffer containing 0.5 mM K-ferricyanide and 0.5 mM K-ferrocyanide). Afterwards, seedlings or adult plants were put in GUS assay buffer (GUS preincubation buffer containing 2 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-gluc)) and incubated for 5 and 16 h, respectively, at 37°C in the dark. The reaction was stopped by washing the seedlings or adult plants 3 times in phosphate buffer for 10 min. Before transfer to microscopic slides, seedlings were incubated in lactic acid to clear the plant material. Adult plants were incubated overnight in 70% EtOH at room temperature to remove the chlorophyll. Plants were stored in 100% EtOH.

This protocol was also followed for tobacco, for each construct 5 independent transgenic lines of the T₁ generation were used. GUS expression was analyzed at different stages of development in the presence and absence of JAME. Plants expressing the β-glucuronidase gene under the control of the 35S CaMV promoter were used as a positive control.

Microscopic analysis was performed on a Nikon eclipse TE2000-e epifluorescence microscope (Nikon Belux, Brussels, Belgium) using a 4x, 10x and 60x Plan Apo objective lens (NA of 0.13, 0.30 and 0.95 respectively) coupled with a standard Nikon CCD camera. Seedlings and plant sections were imaged using the software packages NIS-Elements and Photoshop, and analyzed using ImageJ.

Fluorometric quantification of GUS activity was carried out with five-week-old tobacco plants, essentially as described by Jefferson (1987). The GUS activity is expressed as pmol of 4-methylumbelliferone MU/mg protein/min.

2.6.9 Embedding method for transverse sections

The protocol for embedding Arabidopsis seedlings was adapted from Beeckman *et al.* (1999) and De Smet *et al.* (2003). Briefly, after GUS staining, seedlings were fixed and dehydrated. Subsequently the samples were infiltrated with the resin Technovit 7100 (Klinipath, Olen, Belgium). The tissue was gradually infiltrated using solutions of resin diluted to 30%, 50% and 70% with 95% ethanol (2 hours each). Afterwards they were transferred to 100% infiltration solution for overnight incubation. After vacuum infiltration, another 2 hour incubation with 100% infiltration solution was performed.
Chapter 2: Jasmonate response of the *Nicotiana tabacum* agglutinin promoter in *Arabidopsis thaliana* and *Nicotiana tabacum*

For plant material from seedlings, a two-step embedding method was necessary. In a first step the sample was pre-embedded in a self-constructed flat embedding mould as described by Beeckman *et al.* (1999). The actual embedding was performed in a safe-lock tube filled with freshly made embedding solution. After polymerisation samples are ready for sectioning on the Leica RM 2265 microtome (Leica, Nussloch, Germany).

### 2.6.10 Agglutination assays

To check for lectin activity in tissue extracts, agglutination assays were performed in glass tubes by mixing 10 μl of protein solution with 10 μl of 1 M ammonium sulfate and 30 μl of a 2% solution of trypsin-treated rabbit erythrocytes (BioMérieux, Marcy l’Etoile, France) (made up in phosphate buffered saline containing 137 mM NaCl, 8 mM Na₂HPO₄·2H₂O, 3 mM KCl, 1.5 mM KH₂PO₄, pH 7.4). Agglutination was assessed visually after 10 min at room temperature.

### 2.6.11 Western blot analyses

Protein extracts from tobacco leaves were analyzed by SDS-PAGE in 15 % acrylamide gels. Immunoblot analysis was performed by blocking the membrane first with Tris-buffered saline (TBS; 10 mM Tris-HCl, 150 mM NaCl, 0.1 % (v/v) Triton X-100, pH 7.6) containing 5 % (w/v) non-fat milk powder, followed by consecutive incubation in TBS supplemented with a primary rabbit antibody directed against Nictaba (diluted 1:80), a horseradish peroxidase-coupled goat anti-rabbit IgG (diluted 1:300, Sigma-Aldrich, Bornem, Belgium) and the peroxidase-anti-peroxidase complex (diluted 1:400, Sigma-Aldrich). The primary rabbit antibody directed against Nictaba was obtained by subcutaneous injection of an adult rabbit with Nictaba purified from tobacco leaves, as described by Chen *et al.* (2002). Immunodetection was achieved using a 0.1 M Tris-HCl buffer (pH 7.6) containing 700 μM diaminobenzidine and 0.03 % (v/v) H₂O₂. The reaction was stopped by washing the membrane with distilled water.

### 2.6.12 ELISA assay

The Nictaba content in protein extracts from tobacco leaves and roots was quantified using an ELISA assay with a specific antibody directed against Nictaba, as described by Vandenborre *et al.* 2009a.
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2.6.13 Software packages


BioEdit 7.0.4.1: [www.mbio.ncsu.edu/BioEdit/bioedit.html](http://www.mbio.ncsu.edu/BioEdit/bioedit.html)

BoxShade 3.21: [http://www.ch.embnet.org/software/BOX_form.html](http://www.ch.embnet.org/software/BOX_form.html)


PLACE 28.0: [http://www.dna.affrc.go.jp/PLACE/signalscan.html](http://www.dna.affrc.go.jp/PLACE/signalscan.html)

Chapter 3

Localization of the tobacco lectin in plants and cell cultures
Chapter 3: Localization of the tobacco lectin in plants and cell cultures

3.1 Abstract

Nictaba is a carbohydrate-binding protein that is expressed in the leaves of *N. tabacum* plants only after treatment with jasmonates and insect herbivory. Molecular cloning and sequence analysis revealed the absence of a signal peptide and the presence of a typical monopartite nuclear localization signal (NLS) $^{102}$KKKK$^{105}$, suggesting that the protein could locate to the nucleus. Immunolocalization studies revealed the nucleocytoplasmic localization of Nictaba. Previous localization studies using EGFP-tagged fusion proteins indicated that the NLS is required and sufficient for transporting Nictaba from the cytoplasm into the nucleus. All these studies were performed by transient transformation of tobacco Bright Yellow BY-2 suspension cells using particle bombardment.

In this chapter, the localization studies were extended to stably transformed suspension cells as well as transiently and stably transformed *N. benthamiana* leaf cells. Three different EGFP constructs were included in the analysis, being the native Nictaba sequence, an NLS mutant ($^{102}$KTAK$^{105}$) and a mutant affected in the carbohydrate binding site (W$^{15}$→L$^{15}$). No differences were observed between suspension cells and leaf cells being transiently or stably transformed with the different EGFP constructs. Unexpectedly, the localization pattern for Nictaba and the two mutant proteins was similar, indicating that neither the NLS nor the carbohydrate binding activity of the protein are necessary for nuclear import.

3.2 Introduction

In 2002, Chen et al. reported the purification and characterization of the *N. tabacum* lectin, called Nictaba, from jasmonate-treated tobacco leaves. Immunocytochemical analyses using an antibody specifically directed against the lectin showed that Nictaba is exclusively present in the cytoplasm and the nucleus of the parenchyma cells. No lectin was detected in the vacuole or the chloroplasts, or in the vascular tissues. This subcellular localization is in good agreement with the absence of a signal peptide on the lectin polypeptide translated from the cDNA, and the presence of a nuclear localization signal ($^{102}$KKKK$^{105}$) (Chen et al. 2002). Later, localization studies were also performed in transiently transformed tobacco Bright Yellow 2 (BY-2) cells expressing an EGFP-Nictaba fusion protein after particle bombardment. A strong fluorescence was visible in the cytoplasm and the nucleus of the cell, 24 h after biolistic delivery. Interestingly, 48 h after bombardment, the EGFP fluorescence in the nucleus was no longer evenly distributed. EGFP-Nictaba fluorescence was more concentrated at the periphery of the nucleus. Mutation of the NLS sequence into $^{102}$KTAK$^{105}$ changed the localization pattern for the mutant lectin coupled to EGFP in that the fusion protein could only be...
detected in the cytoplasm and was no longer observed inside the nucleus, suggesting that the sequence $^{102}$KKK$^{105}$ is required for transport into the nucleus (Lannoo et al. 2006b).

In planta localization studies using transformed N. tabacum plants overexpressing the EGFP-Nictaba fusion protein were not successful. Although EGFP expression was observed in the first two weeks after seed germination, the fluorescence rapidly decreased in older plantlets. Since these T$_1$ generation plants still contained the transgene, the most likely explanation for this phenomenon was gene silencing (Lannoo 2007b).

In this study, we have analyzed the localization of Nictaba in N. benthamiana plants and tobacco BY-2 suspension cells. It was shown before that, in contrast to many tobacco species and cultivars, N. benthamiana does not express a Nictaba homologue, simply because N. benthamiana is lacking a sequence homologous to Nictaba (Lannoo et al. 2006a). Similarly, no Nictaba sequence could be amplified from genomic DNA extracted from BY-2 cells. Therefore, the use of N. benthamiana plants or BY-2 cells should enable to circumvent the problem or reduce the risk of post-transcriptional gene silencing.

Both transient and stable transformation of N. benthamiana plants were performed using an Agrobacterium-based transformation protocol. Both methods have their advantages and disadvantages. Transient transformation is a very fast protocol which yields results within one week, it is also an easy tool for performing co-localization studies. In contrast to transient transformation, stable transformation allows the accumulation of the fusion protein over a much longer time period. However, it takes up to six months before any results can be obtained. Next to localization studies the stably transformed seedlings can also be used for more advanced microscopic analyses, like fluorescence recovery after photobleaching (FRAP). For these experiments it is important that the decrease in fluorescence intensity is due to laser application and not due to decay of fluorescence.

Next to the stable transformation of plants, stable transformation was also performed for BY-2 cell cultures. Tobacco suspension cells were transformed using the pathogenic bacterium A. tumefaciens harboring a Nictaba construct coupled to EGFP. Similar to plants, BY-2 suspension cells expressing the fusion protein can be used for localization experiments, but also FRAP experiments or drug-treatment are feasible.

The localization patterns obtained for three different Nictaba constructs, comprising the native Nictaba sequence, an NLS mutant ($^{102}$KTAK$^{105}$) and a mutant affected in the carbohydrate binding site (W$^{15}$$\rightarrow$L$^{15}$) covalently coupled with EGFP, were compared between plants and cell cultures.
Chapter 3: Localization of the tobacco lectin in plants and cell cultures

3.3 Results

3.3.1 Localization study of the tobacco lectin

The localization pattern was studied and compared for different Nictaba constructs, including the native Nictaba sequence, its NLS mutant and a lectin sequence that was mutated in the carbohydrate binding site, further referred to as ∆CRB-Nictaba (Table 3.1). Previously, it was show that residue Trp15 plays an essential role for the sugar binding activity of the lectin, since mutation of this residue into Leu caused complete loss of lectin activity (∆CRB-Nictaba) (Schouppe et al. 2010). The fluorescence pattern for each of the EGFP fusion constructs was analyzed using stable and transiently transformed N. benthamiana plants, and stably transformed tobacco BY-2 suspension cells. Localization patterns were visualized using confocal microscopy.

Table 3.1 Overview of the different Nictaba sequences and EGFP fusion proteins under study.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
<th>Construct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nictaba</td>
<td>Wild type</td>
<td>EGFP</td>
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<tr>
<td></td>
<td></td>
<td>Nictaba</td>
</tr>
<tr>
<td></td>
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<td>Nictaba</td>
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<tr>
<td></td>
<td></td>
<td>EGFP</td>
</tr>
<tr>
<td>∆NLS-Nictaba</td>
<td>Nuclear localization signal</td>
<td>EGFP</td>
</tr>
<tr>
<td></td>
<td>102KKK&lt;sup&gt;105&lt;/sup&gt;→102KTAK&lt;sup&gt;105&lt;/sup&gt;</td>
<td>∆NLS-Nictaba</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EGFP</td>
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<td></td>
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<td>Nictaba</td>
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<td></td>
<td></td>
<td>∆NLS-Nictaba</td>
</tr>
<tr>
<td>∆CRB-Nictaba</td>
<td>Carbohydrate binding site</td>
<td>EGFP</td>
</tr>
<tr>
<td></td>
<td>W&lt;sup&gt;15&lt;/sup&gt;→L&lt;sup&gt;15&lt;/sup&gt;</td>
<td>∆CRB-Nictaba</td>
</tr>
</tbody>
</table>
3.3.2 Nucleocytoplasmic expression of the native Nictaba protein fused with EGFP

3.3.2.1 Nictaba is similarly expressed in tobacco plants and suspension cells

Both the C- and N-terminal fusion constructs between the EGFP and Nictaba sequences were made. However, after transient transformation of *N. benthamiana* plants no expression was visible for the Nictaba-EGFP fusion protein using confocal microscopy. This experiment was repeated several times but no fluorescence was observed. Therefore, the different mutant Nictaba sequences were tagged with EGFP at the N-terminus but not at the C-terminus.

Expression of the native Nictaba protein fused to the C-terminus of EGFP was observed within the nucleus and the cytoplasm of cells of stably transformed *N. benthamiana* plants. EGFP-Nictaba expression was seen for the following cell types: trichomes, epidermis cells, mesophyll cells and stomata (Figure 3.1). A plot profile through the nucleus shows a high fluorescence intensity at the nuclear edges. There is a clear drop in fluorescence inside the nucleus, but the intensity is still at a higher level than in the bulk of the cell (Figure 3.1 D). Similarly, after transient transformation of *N. benthamiana* leaves the lectin was also detected in the nucleus and the cytoplasm of the cell (Figure 3.2 A). The plot profile shows a highly similar pattern as seen for the stably transformed leaf cells, although the drop in fluorescence intensity inside the nucleus is less profound (Figure 3.2 B). Similar results were also obtained for stably transformed BY-2 suspension cells (Figure 3.2 C and D).

The fluorescence patterns observed for the different expression systems are very similar and are in agreement with previously obtained results. Nictaba is clearly expressed in the cytoplasm and the nucleus of the cell after stable and transient transformation. Within the nucleus the expression is much higher at the nuclear rim and drops in the center of the nucleus, although the expression in the nucleus is clearly higher compared with the expression in the vacuole of the cell. This localization pattern was confirmed for the different cell types (epidermis cells, trichomes and stomata).

The green fluorescent protein was used as a control in parallel transformation experiments of *N. benthamiana* plants and tobacco BY-2 suspension cells. EGFP is a 26.9 kDa protein and can freely diffuse to the nucleus. This was very clearly observed in leaf cells of *N. benthamiana* plants as well as in BY-2 suspension cells (data not shown). Plot profiles showed an even distribution of fluorescence all over the nucleus for both *N. benthamiana* epidermal plant cells (Figure 3.3) and BY-2 suspension cells (data not shown).
Figure 3.1 Localization of fusion protein EGFP-Nictaba in stably transformed *N. benthamiana* plants. EGFP-Nictaba expression is shown in the trichomes (A), mesophyll cells (B), epidermis cells (C) and stomata (inset). A plot profile (D) shows the fluorescence intensity within the nucleus (yellow line, C). Autofluorescence originating from the chloroplasts is shown in red. Scale bars are 25 µm, unless stated differently.
Figure 3.2 Localization of fusion protein EGFP-Nictaba in transiently transformed *N. benthamiana* epidermis leaf cells (A) and stably transformed tobacco BY-2 suspension cells (C). Plot profiles show the fluorescence intensity within the nucleus (yellow line, A and C) of epidermis (B) and BY-2 cells (D), respectively.
Figure 3.3 Localization of free EGFP in transiently transformed \textit{N. benthamiana} epidermis leaf cells (A). A plot profile shows the fluorescence intensity within the nucleus (yellow line, A) of epidermis cells (B).

3.3.2.2 Validation of the transformation with Nictaba EGFP fusion proteins

Next to microscopic analyses to check the fluorescence distribution in the plant cells and tissues, the presence of the EGFP transgene in the transformed tissues was also confirmed by Western blot. This analysis can confirm the expression of the EGFP construct and in addition allows checking if the fusion protein was degraded and lost its fluorescent tag. Therefore, Western blot analysis allows confirming that the microscopy images show the fluorescence of the tagged lectin and not of the free EGFP.

Protein was extracted from stably transformed \textit{N. benthamiana} plants of the T\textsubscript{2} generation expressing the EGFP-Nictaba construct. Leaf extracts were used to perform a Western blot analysis using antibodies directed against Nictaba. All tested plants were expressing the EGFP-Nictaba fusion protein and it could be confirmed that these fusion proteins were still intact (Figure 3.4 A). After transformation of tobacco BY-2 suspension cells, fluorescent calli were selected to establish a liquid culture. After several weeks of cell culturing, transformation of the cells was checked by microscopy and Western blot analysis of cell extracts using antibodies directed against Nictaba (Figure 3.4 B). In both blots (Figure 3.4 A and B), a clear band is visible at 48 kDa, the size of the EGFP-Nictaba fusion product, indicating that transformed plants and cells contain a fusion protein with fluorescent tag. Recombinant Nictaba was used as a positive control and has a molecular weight of 19 kDa. The faint band present in the lane marked ‘rNictaba’ corresponds to a dimerization product of Nictaba of approximately 38 kDa.
Figure 3.4 Western blot analysis on cell material extracted from stably transformed *N. benthamiana* (A) or stably transformed tobacco BY-2 cells (B) expressing the EGFP-Nictaba construct using an antibody directed against Nictaba. 10 µg (A) and 50 µg (B) of EGFP-Nictaba was loaded. Recombinant Nictaba (1 µg) is used as a positive control (rNictaba) on the blot.

Similar Western blot analyses were also performed with leaves of transiently transformed tobacco plants infiltrated with an Agrobacterium construct harboring the Nictaba sequence fused to EGFP. Two days after Agrobacterium infiltration, leaf material was collected and analyzed to check whether the protein coupled with EGFP was still intact. The Western blots show a clear expression of the control EGFP and EGFP N-terminally coupled with Nictaba using antibodies directed against Nictaba or EGFP (Figure 3.5 A and B). The C-terminally tagged Nictaba does not yield such a clear result. Using an antibody directed against EGFP, a faint band with the correct size can be distinguished, although Western blot detecting with antibodies directed against Nictaba gave no good result.
Figure 3.5 Western blot analysis on leaf material extracted from transiently transformed *N. benthamiana*. Nictaba N- and C-terminally tagged with EGFP as well as free EGFP (control) were transiently expressed in leaf epidermis cells. Detection was performed using an antibody directed against EGFP (A) and Nictaba (B). Recombinant EGFP (A, rEGFP 1 µg) and recombinant Nictaba (B, rNictaba 0.1 µg) were used as positive controls on the blots. Equal amounts of protein (10 µg) were loaded in each lane (EGFP, EGFP-Nictaba and Nictaba-EGFP).

To explain this unexpected result, analyses were also performed at RNA level. RNA extraction of transiently transformed leaf material was performed and used for cDNA synthesis. PCR reactions using primers to amplify the Nictaba gene sequence only, the Nictaba gene N-terminally tagged with EGFP and the Nictaba gene C-terminally tagged with EGFP confirmed the presence of both mRNAs encoding EGFP-Nictaba and Nictaba-EGFP (Figure 3.6). Actin was also amplified as a control for cDNA quality. As a positive control, plasmid DNA of the corresponding vectors was used. The negative control was a no template control, where no DNA was added to the PCR mixture.
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Figure 3.6 RT-PCR on leaf material extracted from transiently transformed *N. benthamiana* plants. Constructs containing the sequences for N- and C-terminal fusion of Nictaba with EGFP were transiently expressed. RNA was extracted after 2 days and cDNA was synthesized. The different cDNA samples were amplified with Nictaba-specific primers (amplification product 498 bp), primers amplifying the Nictaba gene with N-terminally coupled EGFP (amplification product 1038 bp), primers amplifying the Nictaba with C-terminally coupled EGFP (amplification product 1236 bp) and actin primers (amplification product 722 bp) to check for cDNA quality. For every construct, the corresponding plasmid DNA was used as a positive control (●). No template was used in the negative control (○).

### 3.3.2.3 Confirmation of the nucleocytoplasmic location using organelle markers

Co-localization studies were performed with different marker proteins to facilitate elucidating the exact subcellular localization of Nictaba. Therefore, the coding sequence of proteins with a clearly determined subcellular localization was covalently coupled with the sequence for a fluorescent tag. ABC-transporter ABCI.21 and NUP62 were both N- and C-terminally tagged with RFP and were previously shown to be located in the cytoplasm and the nuclear membrane of the cell, respectively.

#### Cytosolic localization

ABC-transporter At5g44110.1 is localized in the cytosol (cytoplasmic strands) and around the nucleus (Marin *et al*. 2006). It functions as a transporter protein using ATP. The ABC-transporter-RFP fusion protein was co-expressed with EGFP-Nictaba in *N. benthamiana* epidermal leaf cells by infiltration of a mixture of Agrobacterium cells (1:1), harboring constructs for EGFP-Nictaba and RFP-ABC transporter (Figure 3.7). Both proteins are expressed in the cytosolic compartment of the cell. Similar results were obtained for the C-terminal fusion of RFP with the ABC-transporter.
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Figure 3.7 Confocal microscopy images of transiently transformed N. benthamiana leaves co-expressing EGFP-Nictaba and RFP-ABC transporter. Panel A and B show the individual signals of EGFP-Nictaba and RFP-ABC transporter, respectively. Panel C shows the overlay image of both fluorescent signals.

An alternative method to examine whether a protein is localized within the cytosol is by protoplast formation and subsequent lysis of plant protoplasts using a nonionic detergent such as Triton X100. Protoplasts were made from stably transformed BY-2 cells, expressing EGFP-Nictaba (Figure 3.8 A). Figure 3.8 A clearly shows that the lectin is expressed in the nucleus and the cytoplasm of the cell. To check if Nictaba is also localized in the cell membrane, different concentrations of Triton X100, ranging from 0.1 to 0.5 percent, were added and lysis of the protoplasts was monitored by confocal microscopy (Figure 3.8 B). Once the cell membrane is degraded, the fluorescence gradually escapes from the protoplast and no fluorescence remains, thus indicating that Nictaba is not associated with the cell membrane and only resides within the cytoplasm.
Figure 3.8 Confocal images of stably transformed BY-2 protoplasts expressing EGFP-Nictaba (A) and treatment of these protoplasts with 0.1% Triton X100 (B). A-1 shows the green fluorescence signal, A-2 the transmission signal and A-3 the overlay image. B-1 shows the overlay image of the protoplast right after adding the detergent, B-2 shows the moment when the cell membrane breaks and fluorescence escapes and B-3 shows a damaged protoplast from which almost all fluorescence has disappeared. (n, nucleus; c, cytoplasm).

Nuclear localization

The nuclear envelope (NE) forms a physical barrier between cytosol and nucleoplasm. Direct communication between these two compartments is controlled by nuclear pore complexes (NPCs), which are distributed in the NE (Fiserova et al. 2009). Little is known about the plant nuclear pore system, but recently it was shown that the plant NPC contains at least 30 nucleoporins (Tamura et al. 2010). Tamura et al. isolated At2g45000 and characterized it as the homolog of human nucleoporin NUP62. NUP62 is a structural constituent of the nuclear pore complex and is present in the central channel (Xianfeng and Meier 2007). NUP62 was fused with RFP for co-localization studies, but unfortunately, no fluorescence was observed after transient transformation in N. benthamiana epidermal leaf cells.
3.3.3 Localization pattern of the Nictaba mutant affected in its carbohydrate binding activity

Transient transformation of the carbohydrate binding mutant of Nictaba (ΔCRB, Table 3.1) in *N. benthamiana* leaves shows the presence of the protein in the nucleus and the cytoplasm of the plant cell (Figure 3.9 A). A plot profile clearly shows the higher fluorescence intensity at the edges of the nucleus compared to the center of the nucleus, which still has a higher fluorescence intensity compared to the surrounding organelles (Figure 3.9 B). Stable transformation of the mutant in tobacco BY-2 suspension cells yielded similar results (Figure 3.9 C). Again, the expression is stronger at the nuclear rim, lower in the center of the nucleus and almost negligible in the surrounding cell compartments (Figure 3.9 D).

The ΔCRB Nictaba only differs from wild-type Nictaba in its ability to bind sugars. The mutant protein no longer has the capacity to bind any sugars. Apparently, this lack of sugar-binding activity does not interfere with the localization of the protein in the cell. As seen in the different pictures, the Nictaba CRB mutant also localizes to the cytoplasm and the nucleus, and similar to wild-type Nictaba is located primarily at the nuclear rim (Figure 3.9).
Figure 3.9 Localization of fusion protein EGFP-ΔCRB-Nictaba in transiently transformed *N. benthamiana* epidermis leaf cells (A) and stably transformed tobacco BY-2 cells (C). The plot profiles show the fluorescence intensity within the nucleus (yellow line, A and C) of epidermis (B) and BY-2 cells (D), respectively. Scale bars are 50 µm.
3.3.4 The NLS mutant of Nictaba localizes to the nucleus and the cytoplasm of the cell

Expression of the NLS mutant is observed in stably transformed *N. benthamiana* plants in the cytoplasm but surprisingly also in the nucleus of the plant cell. This pattern was similar for all the different cell types (Figure 3.10) and confirmed by the plot profiles. The plot profile as shown in figure 3.10 D is very similar to the one seen for the native protein. A higher fluorescence intensity is visible at the edges of the nucleus compared to the fluorescence inside the nucleus, which is still higher compared to the fluorescence in the rest of the cell (Figure 3.10 D). Transient expression of the NLS mutant in tobacco leaves yielded comparable results (Figure 3.11 A). The mutant protein is present in both the cytoplasm and the nucleus, again with a higher expression at the nuclear rim (Figure 3.10 B). This localization pattern was also seen in stably transformed BY-2 cells (Figure 3.11 C and D).

Fluorescence patterns representing the expression of the NLS mutant yielded very similar results in the transformed tobacco plants and suspension cells. Furthermore, the localization of ∆NLS-Nictaba is similar to the localization of wild-type Nictaba. The plot profiles show that the Nictaba protein with the mutation in the nuclear localization signal still has the opportunity to enter the nucleus. Its expression is also more confined to the nuclear rim. However, these results are different compared to previous research, where the EGFP-∆NLS-Nictaba construct was introduced into the cell through particle bombardement (Lannoo *et al.* 2006b).
Figure 3.10 Localization of fusion protein EGFP-ΔNLS-Nictaba in stably transformed *N. benthamiana* plants. EGFP-ΔNLS-Nictaba expression is shown in the trichomes (A), mesophyll cells (B), epidermis cells (C) and stomata (inset). A plot profile (D) shows the fluorescence intensity within the nucleus (yellow line, C). Autofluorescence originating from the chloroplasts is shown in red. Scale bars are 25 µm, unless stated differently.
Figure 3.11 Localization of fusion protein EGFP-ΔNLS-Nictaba in transiently transformed *N. benthamiana* epidermis leaf cells (A) and stably transformed tobacco BY-2 cells (C). The plot profiles show the fluorescence intensity within the nucleus (yellow line, A and C) of epidermis (B) and BY-2 cells (D), respectively. Scale bars are 50 µm.
3.3.5 Nictaba is a highly dynamic protein

To test if Nictaba can dynamically shuttle between the nucleus and the cytoplasm, the localization of the fusion protein EGFP-Nictaba was analyzed in tobacco BY-2 suspension cells after treatment with leptomycin B (LMB). LMB has been shown to specifically inhibit nuclear export signal-mediated export by interacting with the export receptor Exportin 1 from A. thaliana, AtXPO1 (Haasen et al. 1999) and was also successfully used in tobacco BY-2 cells (Dong et al. 2005). Different concentrations of LMB, ranging from 20 to 100 nM, were used and nuclear localization of EGFP-Nictaba was followed for 3.5 hours. Every half hour, nuclear fluorescence was checked but no significant accumulation of EGFP-labeled protein inside the nucleus or depletion in the cytoplasm could be detected (data not shown).

To assess the molecular dynamics of the tobacco lectin within the nucleus, FRAP analysis was performed in stably transformed tobacco BY-2 suspension cells expressing the EGFP-Nictaba fusion protein. Fluorescent molecules were permanently bleached at a 4 μm diameter spot inside the nucleus and the rate of fluorescence recovery was measured. If Nictaba is bound to other structural components of the nucleus, FRAP recovery would be retarded compared to proteins which are moving freely and are not hindered. Fluorescence recovery was evaluated by comparing the FRAP recovery of the fusion protein EGFP-Nictaba to that of unconjugated EGFP. Judging from the fluorescence recovery profile, both EGFP-Nictaba and EGFP behaved in a similar manner (Figure 3.12). However, using the same settings and a bleaching region of the same size, the bleaching of free EGFP was less efficient (5% bleached) presumably due to rapid diffusion of EGFP molecules to the bleached region. Also the fusion protein EGFP-Nictaba was difficult to bleach (7% bleached) indicating that it is a highly dynamic protein within the nucleus but diffuses less fast than free EGFP.
3.4 Discussion

The localization of the wild-type Nictaba protein and two mutants, one affected in the nuclear localization signal and one affected in the carbohydrate binding activity, was determined. Enhanced green fluorescent protein (EGFP) was used to tag the different Nictaba variants under study. EGFP differs from wild-type GFP, originating from *Aequorea victoria*, by a greater folding efficiency at 37°C and a single excitation peak at 490 nm. EGFP has a traditional β-barrel structure with the chromophore, Thr<sup>65</sup>-Tyr<sup>66</sup>-Gly<sup>67</sup>, located in the core of the protein (Arpino *et al.* 2012).

Previously, *Chen et al.* (2002) showed that Nictaba is expressed in the nucleus and the cytoplasm of the leaf cells of jasmonate-treated *N. tabacum* plants using an immunocytochemical approach (Chapter 1, 1.1.5). *Lannoo et al.* (2006b) confirmed that EGFP-Nictaba is a functional lectin by checking the lectin activity using a binding assay. EGFP-Nictaba specifically bound to a mixture of GlcNAc-oligomers which was shown to be efficient for the purification of native Nictaba from tobacco leaves. In addition, the fusion protein could agglutinate rabbit erythrocytes indicating that EGFP-Nictaba is a fully functional lectin.

Localization studies were performed in different systems, namely transformed plants and transformed suspension cells and a detailed comparison was made. Tobacco BY-2 suspension cells are quite easy to transform, deliver a ready-to-use line and are easy to image. But these cells have to be transferred into fresh medium every week, which means a risk for contamination or loss of the
culture. Furthermore, there is always a difference with the *in planta* situation, as there are no distinguishable structures and cells are completely separated from each other. Nevertheless, BY-2 cell cultures are a very useful tool.

Transformation of *N. benthamiana* plants was performed using both transient and stable techniques. Compared to cells, the tobacco plant gives a more realistic image of the exact localization of the proteins. Considering this, stable transformation is the best option but also the most labor-intensive and time-consuming method. The big advantage of transient transformation is the fast generation of results. At the same time transient transformation is a very useful tool to co-transform different constructs. Though transient transformation is the most artificial of all the presented methods, it can be used to check the expression of the construct, but preferably the results should be confirmed by other techniques.

For all N-terminally tagged proteins, the fusion proteins were expressed and yielded a clear fluorescence signal under the confocal microscope. Unfortunately, Nictaba-EGFP expression was never visualized after transformation, although RNA and protein were detected in the transformed tissues. Possibly, a problem occurred during protein folding. In the three-dimensional model for Nictaba, the C-terminal sequence of Nictaba is more buried in the folded polypeptide compared to the N-terminal extremity which is far from the core of the protein and therefore more easily accessible (Schouppe et al. 2010). Therefore fusion of EGFP to the C-terminus of Nictaba might hamper proper protein folding, which can explain the lack of fluorescence for Nictaba-EGFP.

Confocal microscopy of the transformed cells and plants confirmed the localization of Nictaba in the nucleus and cytoplasm of the cell. Within the nucleus a higher expression of Nictaba was observed at the periphery near the nuclear envelope. Regardless of the transformation method or cell type used, this specific pattern was always confirmed.

Ever since the first report by Chen et al. in 2002, Nictaba is considered as a nucleocytoplasmatic lectin. Immunochemistry as well as particle bombardment of beads coated with plasmid DNA expressing EGFP-Nictaba confirmed partial localization of Nictaba in the nucleus. Analysis of the cDNA sequence encoding Nictaba revealed the presence of a classical monopartite NLS sequence, namely KKKK. The classical nuclear import pathway is the best characterized pathway for nucleocytoplasmic transport. In this pathway, the adaptor protein importin α directly binds the classical NLS (cNLS) of the protein to be imported. Importin α forms a heterodimeric import receptor together with the β-karyopherin importin β. Importin β mediates interactions with the nuclear pore complex (Lange et al. 2007).
The NLS of Nictaba fits the key requirements for a classical NLS. It consists of a monopartite stretch of basic amino acids which fits the consensus sequence of K(K/R)X(K/R). However, based upon primary sequence analysis alone, it is impossible to decide whether a NLS is functional. The sequence must be necessary for import, implicating a serious hindrance when it is altered. In contrast to previous research (Lannoo et al. 2006b), a localization pattern with expression in both the nucleus and the cytoplasm was seen for the Nictaba mutant with an altered NLS. The transformation of the NLS mutant was repeated several times in both cells and plants, always resulting in the same localization pattern. At present, we cannot explain the different localization patterns observed after particle bombardement and plant/cell transformation of the same construct. Although a cNLS is present but not functional, nuclear localization through alternative mechanisms is also likely to be prevalent, for example by passive diffusion of small proteins (<40 kDa) through nuclear pore complexes or by a non-classical nuclear import via importin β-like receptors (Lange et al. 2007, Seibel et al. 2007).

The Nictaba mutant lacking carbohydrate binding activity has a very similar expression pattern compared to wild-type Nictaba. There is a clear expression in the cytoplasm and in the nucleus, in particular at the periphery. Thus nuclear import of Nictaba through nuclear pore complexes is independent of the sugar-binding capacity of the lectin.

Tobacco BY-2 suspension cells stably expressing the fusion protein EGFP-Nictaba were used to analyze the mobility of the fusion protein inside the cell. The suspension cells were treated with a drug LMB which disturbs export out of the nucleus by blocking the export receptor Exportin 1. Since blocking of this receptor did not cause an accumulation of fluorescence inside the nucleus and a depletion of fluorescence in the cytoplasm, the tobacco lectin is presumably exported from the nucleus by different export pathways or through passive diffusion. Another technique to study protein mobility is FRAP. Using tobacco BY-2 suspension cells stably expressing EGFP-Nictaba and cells expressing EGFP, the recovery of fluorescence after bleaching was measured. Unfortunately, it was technically not possible to bleach a spot completely and therefore no comparison between recovery time and diffusion coefficient could be performed. However, it was clear that Nictaba recovers fast in FRAP experiments, since no complete bleaching was feasible. Before the software was able to measure a decrease in fluorescence, the bleached spot was already recovering, resulting in a net effect that is small. Fast recovery is typical for many nuclear proteins, suggesting that these proteins are transiently bound to chromatin, since stable binding interactions would result in incomplete or very slow FRAP recoveries (Mueller et al. 2010). Fast recovery is typically seen for transcription factors which exhibit a highly mobile diffusive behavior allowing complete coverage of the nucleus without a need for the cell to invest a lot of energy (Hager et al. 2009).
Independent of the transformation method or system used, Nictaba is always localized in the nucleus and the cytoplasm of the cell. Surprisingly, mutant Nictaba proteins lacking a monopartite NLS were still transported into the nucleus. Similarly the sugar binding capacity of Nictaba is not crucial for nuclear import. These results indicate that Nictaba is not shuttled into the nucleus through the classical import pathway or via its lectin activity but uses alternative mechanisms.

3.5 Materials and methods

3.5.1 Plant material

Seeds of *N. benthamiana* were supplied by Dr Verne A. Sisson (Oxford Tobacco Research Station, Oxford, NC, USA). To establish an *in vitro* culture, dry seeds were surface sterilized with 70% (v/v) ethanol for 2 min, followed by 7% (v/v) NaOCl for 10 min. After rinsing thoroughly with sterile distilled water seeds were sown and germinated on solid Murashige and Skoog medium (containing 4.3 g/L MS micro and macro nutrients containing vitamins (Duchefa), 30 g/L sucrose, pH 5.7 (adjusted with 0.5 M NaOH) and 8 g/L plant agar (Duchefa)). Alternatively seeds were germinated in soil. *In vitro* and *in vivo* grown tobacco plants were cultivated in a growth cabinet or growth chamber, respectively, at 25 °C with a 16/8 h light/dark photoperiod.

*N. tabacum* cv Bright Yellow-2 (BY-2) cells were grown in 250 ml erlenmeyers filled with 40 ml of liquid Murashige and Skoog (MS) medium on an orbital shaker (25°C, 150 rpm, constant darkness). The MS medium (adjusted to pH 5.7 with 1 M KOH) contained 4.3 g/L MS micro and macro nutrients (Duchefa), 30 g/L sucrose, 0.2 g/L KH$_2$PO$_4$ and 40 μl of a 1000-fold concentrated vitamin/hormone stock (containing per ml: 0.4 mg 2.4-D dissolved in ethanol, 1 mg thiamine and 100 mg myo-inositol). Cells were subcultured weekly by pipetting 1 ml of a dense culture into 40 ml fresh MS medium.

3.5.2 Construction of EGFP tagged vectors

The Nictaba cDNA (NCBI accession number AF389848) sequence as well as the Nictaba sequence mutated in the NLS ($^{102}$KKK$^{105}$ changed into $^{102}$KTAK$^{105}$) (Lannoo et al. 2006b) or in amino acid W15 (W15→L15) of the carbohydrate binding site (Schouppe et al. 2010) were cloned into the pK7WGF2.0 and pK7FWG2.0 (Karimi et al. 2002) vector using the Gateway™ Cloning technology of Invitrogen (Figure 3.13). Therefore, the Nictaba sequences were amplified as attB-PCR products in a nested PCR using *Taq* DNA Polymerase (Invitrogen) and primers evd 1/3 (with stop codon) or evd 1/6 (without stop codon) in a first PCR reaction, and primers evd 2 and evd 4 in a second PCR reaction to complete the attB sequences (Appendix A). Using this PCR strategy, the different Nictaba sequences were C- or N-terminally tagged with EGFP. Cycling parameters were as follows: 2 min 94°C, 30x (15 s
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94°C, 30 s 55 °C, 1 min 72°C), 5 min 72°C. In these EGFP fusion constructs the lectin sequence will be expressed under the CaMV 35S promoter.

All attB-PCR products were homogeneously recombined in the pDONR221 vector (Invitrogen) using the Gateway® BP Clonase™ Enzyme mix (Invitrogen). Entry clones were subsequently transferred into E. coli strain Top10 cells using heat shock transformation. Transformants were selected by colony PCR. Plasmid DNA was sequenced with primers evd 386 and evd 387. Finally LR reactions were performed with selected entry clones and the destination vectors pK7WGF2.0 or pK7FWG2.0 using the Gateway® LR Clonase™ Enzyme mix (Invitrogen) to obtain expression clones. These expression clones were transformed via heat shock into E. coli cells. Transformants were selected on LB agar plates containing spectinomycin (75 µg/ml) and screened with PCR, using gene specific primers evd 65/66/108 and EGFP primers evd 594/595. All sequences for the PCR primers are shown in Appendix A.

![Figure 3.13](image)

**Figure 3.13** Schematic representations of the vectors pK7WGF2.0 and pK7FWG2.0 for N- or C-terminal tagging Nictaba with EGFP, respectively. LB and RB, T-DNA border sequences; p35S, CaMV 35S promoter; EGFP, enhanced green fluorescent protein gene; att, Gateway recombination site; ccdB, toxin protein gene; T35S, 35S terminator; Kan, kanamycin resistance gene; Sm/SpR, streptomycin/spectinomycin resistance gene.

### 3.5.3 Stable transformation of *N. benthamiana* leaf disks and selection of transgenic lines

The binary vectors containing the Nictaba EGFP fusions were introduced into *A. tumefaciens* C58C1 PGV4000 cells using cold shock. Transformed cells were selected on LB agar plates containing spectinomycin (75 µg/ml) and used for transformation of *N. benthamiana* leaf disks. Transformed plants were grown until they set seeds. These seeds were selected on MS agar plates containing kanamycin (100 µg/ml). The seedlings from the T2 generation were used for the different experiments.

After shoots and roots emerged from the transformed calli, the integration of the T-DNA into the plant genome was checked by PCR on genomic DNA using the Nictaba and EGFP-specific primers evd 65/66/108 and evd 594/595 (Tm = 55°C). Plantlets that yielded a positive result on DNA level were
subsequently checked at RNA level. Therefore, RNA was extracted from leaf material and transcribed into cDNA. The presence of the Nictaba sequence fused to EGFP was analyzed by PCR using the same conditions as indicated above. cDNA quality was examined using primers evd 282 and evd 283 (Tm = 55°C) to amplify part of the ribosomal protein L25 (Genbank accession number L18908).

After screening on both DNA and RNA level, the T₀ plants were submitted to protein extraction. Leaf extracts were made using 20 mM 1,3-diaminopropane (2.5 ml/g fresh weight). These protein samples were used for Western blot analysis. After separation of the protein extracts by SDS-PAGE in 15% acrylamide gels, immunoblot analysis was performed. The membrane was blocked with Tris-buffered saline (TBS; 10 mM Tris-HCl, 150 mM NaCl, 0.1% (v/v) Triton X-100, pH 7.6) containing 5% (w/v) non-fat milk powder, for one hour. Primary antibodies used were a polyclonal rabbit antibody directed against Nictaba or a primary mouse antibody against EGFP (diluted 1:1000, Roche, Basel, Switzerland). After incubation with the primary antibody directed against Nictaba for 60 minutes, the membrane was subsequently incubated with TBS supplemented with a horseradish peroxidase-coupled goat anti-rabbit IgG (diluted 1:300, Sigma-Aldrich) for 60 minutes followed by incubation with the peroxidase-anti-oxidase complex (diluted 1:400, Sigma-Aldrich) for 45 minutes. In the case of EGFP detection the membrane was incubated with a rabbit anti-mouse IgG labeled with horseradish peroxidase (diluted 1:1000, Dako Cytomation, Glostrup, Denmark) for 60 minutes. Immunodetection was achieved using a 0.1M Tris-HCl buffer (pH 7.6) containing 700 µM diaminobenzidine and 0.03% (v/v) H₂O₂. The reaction was stopped by washing the membrane with distilled water.

3.5.5 Transient transformation of *N. benthamiana* plants

The binary vectors containing the Nictaba EGFP fusions were introduced into *A. tumefaciens* C58C1 pMP90 cells using triparental mating. Transformed cells were selected on YEB agar plates (5 g/L beef extract, 5 g/L peptone, 1 g/L yeast extract, 5 g/L sucrose) containing spectinomycin (75 µg/ml) and gentamycin (20 µg/ml).

Transient expression of the gene constructs was obtained by Agrobacterium infiltration in leaves of 3- to 4-week-old *N. benthamiana* plants grown in soil. The transformed Agrobacterium cells were grown in liquid YEB medium (5 g/L beef extract, 5 g/L peptone, 1 g/L yeast extract, 5 g/L sucrose) containing spectinomycin (75 µg/ml) and gentamycin (20 µg/ml) at 25°C (200 rpm) for two days. Cells were harvested by centrifugation and resuspended in infiltration medium (50mM MES, 2mM Na₂HPO₄, 0.5% glucose, pH 5.6). The wash step was repeated twice, the second time using infiltration medium containing 100 µM acetylsyringone. The cells were diluted to yield a final OD₆₀₀ of 0.1 and infiltrated in the leaf epidermal cells. After infiltration, plants were kept in the growth chamber for 2
days. Microscopic analyses were performed the second day after infiltration. Next to microscopic analyses, protein was extracted from the infiltrated leaves and used for Western blot analysis as described in the previous section (3.5.4).

3.5.6 Stable transformation of *N. tabacum* cv BY-2 cells

Four ml of a 7 day-old dense culture of BY-2 cells was diluted in 40 ml of MS medium and grown for four days at 25°C in the dark on a rotary shaker at 150 rpm. *Agrobacterium tumefaciens* cells of strain LBA4404, were transformed with the expression vectors by triparental mating. Transformed LBA4404 cells were grown in 5 ml YEB medium containing gentamycin (20 μg/ml) and spectinomycin (75 μg/ml) for two days at 25°C (200 rpm). One day before co-cultivation the *A. tumefaciens* cells were diluted 1:100 in 10 ml YEB and further grown under the same conditions. For co-cultivation, 4 ml of 4 day-old BY-2 cells were mixed with various concentrations of bacterial cells and incubated for two days at 25°C. Then, the mixtures were transferred onto MS agar plates containing kanamycin (100 μg/ml), vancomycin (200 μg/ml) and carbenicillin (500 μg/ml), and kept at 25°C in the dark. After approximately two weeks, calli became visible and were transferred to fresh selection plates using a sterile tooth pick. Surviving calli expressing the EGFP fusion constructs were selected using the FLA-5100 fluorescent image analyser (FujiFilm, Sint-Niklaas, Belgium) and grown in liquid medium containing kanamycin (100 μg/ml). Protein was extracted from these stably transformed cells by crushing in liquid nitrogen, extraction in 20 mM 1,3-diaminopropane and lysis using glassbeads. These protein samples were subjected to Western blot analysis as described before (3.5.4).

3.5.7 Microscopic analyses

Two days after infiltration, fluorescence of the lower epidermis of the leaf discs was visualized with the confocal laser scanning microscope Nikon A1R (Nikon Belux) using a 40x Plan Apo objective lens (NA of 0.75). EGFP was excited with a 488 nm line of an argon ion laser, fluorescence emission was detected using the 525 nm emission filter for EGFP and 650 nm for autofluorescence of chlorophyll. Seedlings, cells and leaf disks were imaged using the software package NIS-Elements (Nikon) and analyzed using Fiji (http://fiji.sc/Fiji).

3.5.8 FRAP

FRAP analysis was performed on stably transformed tobacco BY-2 cells expressing EGFP-Nictaba and free EGFP using a confocal laser scanning microscope Nikon A1R (Nikon Belux). The sample was scanned for 10 time points (1.8 sec each) to obtain the overall fluorescence level, then a circular region of 4 μm diameter was bleached at 100% laser power (488 nm) and 100 scans (1.8 sec each)
were taken to monitor recovery. Thirty cells were analyzed for each experiment and the results were normalized and averaged.

3.5.9 Software packages


Fiji: http://fiji.sc/
Chapter 4

O-GlcNAc cycling and expression of Nictaba during cell cycle progression in tobacco suspension cells
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4.1 Abstract

Recently, it was shown that the tobacco lectin is interacting in vitro with the core histone proteins. This interaction occurs by binding of the lectin to the O-GlcNAc modification present on the histones. The O-GlcNAc modification of histones in mammalian cells was discovered very recently and was found to be cell cycle-dependent. Hereupon, the question was raised if this modification also occurs in plants and is cell cycle-related. Therefore, tobacco BY-2 and Xanthi suspension cells were synchronized using aphidicolin drug treatment. Transcript expression of the O-GlcNAc transferase (OGT) gene and the Nictaba gene were monitored through cell cycle progression. Concomitantly, O-GlcNAcylation of histone proteins was studied. In this chapter, we show that plant histones are also modified by O-GlcNAc and that this modification is cell cycle-dependent. Nictaba and O-GlcNAcylated histones presumably interact during S- and/or G2-phase, since at this time they are both present within the nucleus. It is possible that through this interaction, Nictaba can act as a modulator of gene transcription.

4.2 Introduction

O-linked N-acetylglucosamine (O-GlcNAc) is a common post-translational modification of proteins occurring in the nucleus and/or the cytoplasm. O-GlcNAc-cycling is accomplished by two enzymes, O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) responsible for adding and removing the sugar unit, respectively. This PTM has been extensively studied in animal cells and serves as a stress sensor triggering a variety of stimuli such as altering protein function, influencing transcription factors and changing gene expression presumably through epigenetics (Butkinaree et al. 2010; Hanover et al. 2012).

Uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) serves as the donor substrate for OGT and is added on Ser/Thr residues of target proteins. This UDP-coupled sugar is the end product of the hexosamine biosynthesis pathway and is also the precursor for glycolipids, O-linked GalNAc and N-linked glycoproteins. This nutrient-dependent pathway is linked with O-GlcNAc cycling, and this series of reactions is referred to as the hexosamine signaling pathway (HSP). HSP serves as a general sensor of the cellular nutritional status and results in nutrient uptake (anabolism) or protein degradation (catabolism) (Hanover et al. 2010).

O-GlcNAcylation is very similar to phosphorylation in that both PTMs target the same amino acids, are both nutrient-dependent and have a role in cell signaling. These modifications are characterized by an extreme crosstalk and regulate each other in a reciprocal manner. According to the specific
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state of the cell O-phosphate and O-GlcNAc compete or coexist. Different cellular processes are regulated by the interplay between these modifications (Hu et al. 2010; Zeidan and Hart 2010).

Recently, Sakabe et al. (2010b) discovered that among the big pool of O-GlcNAcylated proteins, also histone proteins carry this modification. Using a chemoenzymatic approach, in vitro enzyme assays, co-immunoprecipitation and analytical techniques, the presence of O-GlcNAc on histones was confirmed in HeLa cells. Liquid chromatography (LC)-MS/MS enabled to determine some of the specific O-GlcNAcylation sites, being Thr101 of H2A, Ser36 of H2B and Ser47 of H4, but other sites remain to be identified (Sakabe et al. 2010b). Almost at the same time, the research group of Zhang detected O-GlcNAc-cycling on Ser10 of histone H3, a known phosphorylation site. All histone proteins are glycosylated but H3 is modified to a relatively higher extent compared to H4 and H2B, and H2A only weakly displays O-GlcNAc (Zhang et al. 2011). Also Fong and collaborators validated the O-GlcNAc modification of H3 and identified Thr32 as a novel O-GlcNAc site (Fong et al. 2011).

By now O-GlcNAcylation is considered as part of the histone code, a set of PTM of histones which can influence gene expression in combination with trans-acting factors. Histone O-GlcNAcylation associates both with modifications linked with active and repressed chromatin. OGT and OGA can participate in the epigenetic regulation of gene expression by interacting with epigenetic regulators, histones and histone remodeling complexes (Hanover et al. 2012). For example, the O-GlcNAc modification on Ser112 of H2B is associated with active chromatin (Fujiki et al. 2011). Ten eleven translocation (TET) enzymes TET2 and TET3 interact with OGT, target OGT to the chromatin and regulate glycosylation of H2B Ser112. TET2/3 promote OGT activity and TET2/3-OGT complexes co-localize on active promoters inducing transcriptional activation (Chen et al. 2013; Deplus et al. 2013). OGT can also mediate chromatin repression through interaction with TET1 as well as through O-GlcNAcylation of TET1 (Shi et al. 2013).

O-GlcNAc modification on histones was shown to be cell cycle-dependent, whereby O-GlcNAc levels on histones decrease during mitosis and subsequently return to basal levels in the G1- phase (Sakabe et al. 2010b). O-GlcNAcylation was observed to reduce mitosis-specific phosphorylation and therefore O-GlcNAc removal was proposed as a required checkpoint for entering mitosis during G2/M transition (Fong et al. 2011). These findings are in accordance with the decreased expression of OGT and a decrease in total O-GlcNAcylation level during mitosis (Sakabe et al. 2010a). Although a completely different pattern was observed by Zhang et al., glycosylation decreased at the S-phase and then increased as cells moved into the G2- and M-phase (Zhang et al. 2011). Also the total level of O-GlcNAc is cell cycle-dependent and is crucial for the correct sequence of events leading to cellular division. However, results obtained from studies related to this subject often seem
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contradictory. In *Xenopus* oocytes O-GlcNAc is clearly requested for M-phase entry (Dehnnaut et al. 2007), whereas in HeLa cells OGT protein expression decreases during M-phase (Sakabe et al. 2010a; Slawson et al. 2005). Overall O-GlcNAc levels increase once cells enter the G1-phase and rapidly decrease as the cells progress through the S-phase (Drougat et al. 2012).

Little research has been performed related to the O-GlcNAc modification of plant proteins. This modification was first found on tobacco nuclear proteins. However, in contrast to animal O-GlcNAcylations, which involve the addition of a single O-GlcNAc residue, the modifications reported in plants consisted of an oligosaccharide larger than five O-GlcNAc residues (Heese-Peck et al. 1995; Heese-Peck and Raikhel, 1998). In Arabidopsis two OGTs, SPINDLY (SPY) and SECRET AGENT (SEC) are known. They have overlapping functions necessary for gametogenesis and embryogenesis (Hartweck et al. 2002). In contrast, animals have only one SEC-like OGT encoding multiple splice variants. SEC and SPY have overlapping functions, although sec mutants show few developmental defects. SPY is also a repressor of gibberellin signaling, a positive regulator of the cytokinin response, affects circadian rhythms and is involved in root formation (Butkinaree et al. 2010; Olszewski et al. 2010). The OGA gene is extremely conserved in higher eukaryotes but absent in prokaryotes and yeast, and has not been detected in higher plants. The latter organisms may have developed alternative means for O-GlcNAc removal such as selective proteolysis (Butkinaree et al. 2010; Hanover et al. 2010).

Only a few O-GlcNAc modified plant proteins have been studied in more detail. The phosphorylated capsid protein of the potyvirus *Plum pox virus* was reported as a plant OGT target (Fernández-Fernández et al. 2002; Perez et al. 2006). Similarly the interplay between phosphorylation and glycosylation was shown to be essential for the *N. tabacum* non-cell-autonomous pathway protein1 (Nt-NCAPP1) and the phloem NCAPs, and for their interaction and cell-to-cell movement through plasmodesmata (Toaka et al. 2007). Evidence for some O-GlcNAc modified proteins was also found in crops. For instance, in wheat, vernalization increased the overall level of O-GlcNAc modification of proteins (Xing et al. 2009). Kilcoyne et al. (2009) indicated the possibility of an O-GlcNAc modification on a subpopulation of the 14 kDa protein family of a purified alcohol-soluble rice protein extract. Furthermore, two closely related Arabidopsis class I TCP transcription factors, TCP14 and TCP15, are modified in *E. coli* by the Arabidopsis OGT SEC and have the potential to be O-GlcNAc modified in planta (Steiner et al. 2012). At present none of these studies has confirmed the addition of an O-GlcNAc chain to the protein as previously proposed by Heese-Peck et al. (1995). In contrast, similar to animal proteins, all evidence suggests the addition of a single GlcNAc residue.

In this chapter, the presence of O-GlcNAc on plant proteins was analyzed. In particular, the O-GlcNAc modification of plant histones was investigated in more detail, taking into account that Nictaba was
recently reported to interact with histone proteins through this carbohydrate modification (Schouppe et al. 2011). Furthermore, O-GlcNAc levels were followed throughout the cell cycle to check if this PTM is also cell cycle-dependent in plant cells, as shown for animal cells.

4.3 Results

4.3.1 O-GlcNAc cycling on histone proteins during BY-2 cell cycle progression

4.3.1.1 Cell synchronization and monitoring of cell cycle progression

To study if O-GlcNAcylation of histone proteins changes during cell cycle progression, wild-type BY-2 cells were synchronized at the beginning of the S-phase using aphidicolin treatment. After aphidicolin release, cells re-entered the cell cycle and every hour samples were collected for 14 hours to monitor cell cycle progression and duration of the specific phases by means of flow cytometry and determination of the mitotic index.

Analysis of the DNA content by flow cytometry allowed to corroborate the synchrony of the cells (Figure 4.1). Since N. tabacum is a tetraploid plant, the peaks corresponding to 4C and 8C DNA content were followed. These peaks increase and decrease, respectively, as the cells progress through cell division. These results indicate the start of mitosis approximately eight hours after removal of the aphidicolin block (Figure 4.1 panel 8). In addition, the mitotic index was estimated by counting the ratio of cells in prophase to telophase compared with the total amount of cells (data not shown). Based on these analyses and previous BY-2 synchronization studies reported in literature (Swiatek et al. 2002; Gemperlova et al. 2009) a scheme for cell cycle progression is proposed, as shown below the graphs in Figure 4.1.
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**Figure 4.1** Cell cycle progression of synchronized BY-2 cells monitored by flow cytometric analysis of relative DNA content. Wild-type BY-2 cells were synchronized at the G1/S boundary by aphidicolin treatment. Every hour after aphidicolin removal, samples were collected for 14 hours. 4x and 8x refer to the tetraploid 4C and octaploid 8C DNA content of the cells. A scheme showing the different phases of the cell cycle is shown below the graphs.

### 4.3.1.2 Histones are highly enriched in acid-extracted proteins from BY-2 synchronized cells

After BY-2 cell synchronization, samples were also collected for protein analyses. At 1-h-intervals after removal of aphidicolin, proteins were prepared by acid extraction. Since few proteins will remain in solution using these harsh conditions, it can be assumed that protein preparations will be highly enriched for histone proteins. The protein content of histone protein H3 was examined by Western blot analysis using a specific antibody directed against histone H3 (Figure 4.2 A). H3 is clearly expressed in all samples, the highest concentrations being present during G2- and G1-phase.

In parallel, a similar blot was detected using an antibody specifically directed against the phosphorylation on Ser10 of H3 (Figure 4.2 B). The phosphorylation of histone H3 Ser10 was previously shown to be initiated in late G2-phase and continues to the end of mitosis in mammalian cells. This process of H3 phosphorylation is considered to be conserved in plants (Houben et al. 2007). In our analyses, this post-translational modification of histone H3 was followed, to ascertain the synchrony of the cells and to check whether the histone preparations contain posttranslational
modifications. Figure 4.2 B indicates that phosphorylation of Ser10 on plant histone H3 is present especially at G2- and early M-phase, which is in agreement with Houben’s report.

Figure 4.2 Western blot analysis of protein samples obtained during different stages of cell cycle synchronization. Protein extracts were prepared by acid extraction and detected using an antibody against histone H3 (A) or an antibody directed against the phosphorylation on Ser10 of histone H3 (B). Sample 4 is not shown in the latter analysis. A scheme showing the different phases of the cell cycle is shown between the two blots. Equal amounts of protein (1.5 µg) were loaded in each lane. Data shown are representative for 3 (A) and 1 (B) technical repeat(s).

4.3.1.3 OGT expression in synchronized BY-2 cells

The cycling of O-GlcNAc on proteins is carried out by two enzymes, OGT and OGA, responsible for the addition and the removal of the carbohydrate structure, respectively. To get an idea on the level of O-GlcNAcylation through cell cycle progression, the expression of these enzymes could be determined. High levels of OGT and low levels of OGA would generally result in a higher level of the O-GlcNAc modifications on proteins. Two OGT-related genes have been identified in higher plants, whereas only one protein sequence was retrieved in N. tabacum (Accession No. Q8VXD3/Q8VXD4). Unfortunately, the OGA gene has not been detected in higher plants (Hanover et al. 2012). As a consequence, only the transcript levels of OGT have been analyzed during the time course of the cell cycle in N. tabacum BY-2 suspension cells.

First, transcript levels of OGT and histones H1 and H3, used as positive controls, were determined using a semi-quantitative approach, regular RT-PCR. To follow the activity of OGT during cell cycle progression PCR primers were designed based on the DNA sequence (Accession No. AJ249882). For every time point in the cell cycle, cDNA was prepared which served as template for the RT-PCR. Besides OGT expression, also the accumulation of corresponding mRNAs encoding histone H1
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(Accession No. AB029614) and histone H3 (Accession No. AB015760) was investigated using gene-specific primers. It was shown before that genes encoding histones H1 and H3 are expressed specifically in the S-phase of proliferating tobacco cells (Combettes et al. 1999; Meshi et al. 2000; Costas et al. 2011). As shown in Figure 4.3, histone genes H1 and H3 show the highest expression level in the S-phase whereas highest expression of OGT is found throughout the G2- and M-phase. OGT transcription decreases in the G1- and S-stages of the cell cycle.

![Graph](image)

**Figure 4.3** Transcript levels of histones genes H1 and H3, and OGT during cell cycle progression. H1 and H3 are mainly expressed during the S-phase (transcript length 70 and 119 bp, respectively). O-GlcNAc transferase shows a higher expression during G2- and M-phase (transcript length 74 bp). Equal volumes of PCR amplification products were analyzed on gel. Data shown are representative for 3 technical repeats.

Second, quantitative real-time (qPCR) analysis was performed to obtain quantitative expression data for OGT during cell cycle progression. Again *N. tabacum* histone H1 and histone H3 were included as controls. Internal reference genes CycD3;3 (Accession No. AB015222), CycD3;2 (Accession No. AJ011894) and CdkA;4 (Accession No. AF289467) were chosen because of their stable expression levels throughout the cell cycle (Sorrell et al. 1999; Sorrell et al. 2001; Kawamura et al. 2006). Figure 4.4 shows the expression levels of the different gene transcripts, normalized against time point 2. Samples that show significant up- or down-regulation of a gene are indicated with an asterisk. Genes encoding histones H1 and H3 are clearly up-regulated during the S-phase and are down-regulated during the rest of the cell cycle (Figure 4.4 B and C). Differences in OGT expression throughout the cell cycle were less pronounced compared to the expression patterns observed for the histones (Figure 4.4 A). According to these results OGT expression is significantly up-regulated during the S-, M- and G1-phase. qPCR results for samples taken during the G2-phase were not significantly different compared to time point two.
4.3.1.4 O-GlcNAcylation of histone proteins in synchronized BY-2 suspension cells

OGT transcript levels can give an idea about the activity of the enzyme but it remains to be checked which proteins are being modified due to enzymatic activity. Therefore, Western blot analyses were performed to analyze whether OGT activity correlates with an increase in O-GlcNAcylation of proteins in general, and histones in particular. The histone enriched acid extracted protein samples used in Figure 3.3 were further employed to determine the level of O-GlcNAcylation of histone proteins. Therefore, Western blot analysis was performed using an antibody (CTD 110.6) directed against the O-GlcNAc moiety (Figure 4.5). Based on the molecular mass of the proteins (Table 4.1) and additional Western blot analyses using antibodies directed against H2B, H3 and H4 (data not shown), the lower protein bands below the 17 kDa marker were identified as histone proteins H2A, H2B and H3, whereas the polypeptide for histone H1 was located slightly above the 26 kDa marker polypeptide. It is clear that besides the bands matching to the histone proteins, also other
polypeptides are showing good interaction with the anti-O-GlcNAc antibody, indicating that the corresponding proteins are most probably also modified with O-GlcNAc moieties.

The results indicate an elevated level of O-GlcNAc residues on histone proteins at S/G2 transition and during the M-phase (Figure 4.5). When the data are observed in more detail, some differences between the different histone proteins can be perceived. At S/G2 transition they all show an increase in O-GlcNAc ylation although this phenomenon persists longer at G2 for histone H1 compared to histones H2A, H2B and H3. Figure 4.6 suggests a higher level of O-GlcNAc on histone proteins H1 at the beginning of mitosis, while histones H2A, H2B and H3 contain more O-GlcNAc during mitosis.

Figure 4.5 Quantification of O-GlcNAc levels visualized by Western blot analysis. After cell cycle synchronization histones were prepared by acid extraction and detected using the CTD 110.6 antibody directed against the O-GlcNAc modification. Equal amounts of protein (1.5 µg) were loaded in each lane. Data shown are representative for 3 technical repeats.
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Table 4.1 Calculated molecular masses of the different tobacco histone proteins based on protein sequences obtained from Uniprot or NCBI databases. Post-translational modifications of the protein have not been taken into account when calculating the total molecular mass.

<table>
<thead>
<tr>
<th>Histones</th>
<th>Uniprot/NCBI*</th>
<th>Mass (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>Q9SLS1</td>
<td>29.56</td>
</tr>
<tr>
<td>H2A</td>
<td>BAC53941.1*</td>
<td>15.47</td>
</tr>
<tr>
<td>H2B</td>
<td>A8J6VO</td>
<td>15.44</td>
</tr>
<tr>
<td></td>
<td>P93354</td>
<td>15.96</td>
</tr>
<tr>
<td>H3.1</td>
<td>A7VMF1</td>
<td>15.27</td>
</tr>
<tr>
<td>H3.2</td>
<td>Q76MV0</td>
<td>15.27</td>
</tr>
<tr>
<td>H3.3</td>
<td>Q76N23</td>
<td>15.41</td>
</tr>
<tr>
<td>H4</td>
<td>BAF36442.1*</td>
<td>11.43</td>
</tr>
</tbody>
</table>

4.3.2 Localization of the tobacco lectin in synchronized cells of BY-2 overexpression lines

To check whether the localization pattern of Nictaba is cell cycle-dependent, stably transformed BY-2 cells constitutively expressing the EGFP-Nictaba fusion protein were synchronized at the S-phase using aphidicolin treatment. During 12 hours after aphidicolin removal, samples were collected every hour and subsequently, cell cycle progression was examined by flow cytometry, allowing to analyze the DNA content of the nuclei, after release from the somatic cells. In addition, EGFP-Nictaba expression was visualized using confocal microscopy.

The diagrams show the characteristic peaks of fluorescence emission representing the 4C and 8C DNA content. As shown in figure 4.6, the peak pattern gradually changes as the cell cycle progresses, demonstrating a gradual decrease of the 8C nuclei and an increase of the 4C nuclei (Figure 4.6 panels 7-11). Flow cytometric results suggest the start of mitosis approximately 7 hours after aphidicolin release (Figure 4.6 panel 7).

The distribution of the EGFP-coupled lectin was followed throughout the cell cycle. Figure 4.7 clearly shows that Nictaba is expressed in both the nucleus and the cytoplasm of the BY-2 cell. As the cell cycle progresses, no difference in location could be observed for Nictaba. Western blot analyses confirmed that EGFP is linked to Nictaba, indicating that the confocal images show the localization pattern for the fusion protein (Chapter 3, 3.2.2.2).
Figure 4.6 Flow cytometric analysis of the synchronized transgenic BY-2 cell culture expressing the EGFP-Nictaba fusion protein. Transformed BY-2 cells expressing EGFP-Nictaba were synchronized at the G1/S boundary by aphidicolin treatment and followed for 12 hours after aphidicolin removal. At 1 h intervals cell cycle progression was monitored by analyzing the DNA content of the cells. 4x and 8x refer to the tetraploid 4C and octaploid 8C DNA content of the cells. A scheme showing the different phases of the cell cycle is shown below the graphs.
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Figure 4.7 Confocal microscopic analysis of synchronized N. tabacum BY-2 cells expressing EGFP-Nictaba. Lectin expression was visualized during the different stages of the cell cycle. Scale bar is 50 µm.

4.3.3 Expression of the tobacco lectin during the cell cycle of N. tabacum cv Xanthi cells

4.3.3.1 Cell synchronization of N. tabacum cv Xanthi cells and monitoring of cell cycle progression

Taken into account that the tobacco lectin was shown to interact with histone proteins through their O-GlcNAc modification (Schouppe et al. 2011) and the cell cycle dependency of this carbohydrate modification, it was of great interest to examine the expression of Nictaba through cell cycle progression. However, the BY-2 cells used in previous experiments do not express Nictaba simply because the corresponding Nictaba gene is not present in the genome of the cultured BY-2 cell line (Lannoo et al., 2006a). Therefore, another widely used tobacco cell line, N. tabacum cv Xanthi cells, containing the lectin sequence in its genomic DNA, was used for this experiment. In contrast to BY-2 cells, which have a cell cycle that takes about 12 hours, the cell cycle of N. tabacum cv Xanthi cells lasts much longer and takes approximately 25 hours. Especially the G1-phase is very long and goes on for 10 hours (Nishinari and Syôno, 1986). Consequently, synchronization of N. tabacum cv Xanthi cells has not been reported frequently and only few records are available in literature.

Similar to previous experiments the N. tabacum cv Xanthi cells were blocked at G1/S transition using aphidicolin and after drug removal, cells could progress through the cell cycle. For practical reasons and taken into account that the most interesting phases of the cell cycle correspond to the S, G2 and M-phases (right after aphidicolin removal), samples were analyzed in the first 14 hours after drug removal. Cell cycle progression was followed using flow cytometric analyses, but unfortunately an insufficient amount of nuclei could be extracted and no useful results were obtained. Therefore, the
amount of cells at mitosis was also estimated by analyzing the mitotic index (Figure 4.8 A). A clear peak representing cells in the mitotic phase was observed at 12 hours after aphidicolin removal.

Taken into account the data for the analysis of the mitotic index as well as the data reported in literature a general scheme for cell cycle progression in *N. tabacum* cv Xanthi cells is proposed (Figure 4.8 B).

**Figure 4.8** Cell synchrony of *N. tabacum* cv Xanthi cells was estimated by calculating the mitotic index (A). Below the graph, a scheme shows the different phases of the *N. tabacum* cv Xanthi cell cycle (B).

### 4.3.3.2 Nictaba and OGT expression in synchronized *N. tabacum* cv Xanthi cells

After removal of aphidicolin, samples for RNA and protein analyses were collected from the synchronized *N. tabacum* cv Xanthi cell culture at 1 h intervals, and this for 14 consecutive hours. At every time point, RNA was extracted from the synchronized *N. tabacum* cv Xanthi cells, and transcribed into cDNA. Transcript levels for *Nictaba*, histone *H3* and *OGT* expression were determined using semi-quantitative RT-PCR (Figure 4.9). Histone *H3* was reported to be expressed specifically in the S-phase of synchronized plant cells. In our analyses, however, histone *H3* RNA is expressed in the late S-phase but also in the other phases of the cell cycle. This result is possibly influenced by the larger fraction of non-synchronized cells after aphidicolin treatment (see Figure 4.8). *Nictaba* expression is up-regulated in the S-phase and gradually decreases during the G2-phase. Lectin expression is clearly reduced during the M-phase. *OGT* shows a slightly elevated expression particularly at mid S-phase, during the G2-phase and at the beginning of G1-phase.
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Figure 4.9 Transcript levels of histone H3, Nictaba and OGT during cell cycle progression of synchronized N. tabacum cv Xanthi cells. Histone H3 is used as a control (transcript length 119 bp). Nictaba (transcript length 498 bp) expression during the different phases of the cell cycle was compared to the expression of O-GlcNAc transferase (transcript length 74 bp). Equal volumes of PCR amplification products were analyzed on gel.

4.3.3.3 O-GlcNAcylation of proteins in synchronized N. tabacum cv Xanthi cells

After collection of the acid-extracted proteins, the samples were subjected to Western blotting followed by incubation with an antibody directed against histone H3. The highest concentrations of histone H3 were detected in protein samples taken at the S/G2 transition (Figure 4.10 A). To verify if and which fraction of these proteins are O-GlcNAcylated, a similar blot was incubated with the CTD110.6 antibody directed against the O-GlcNAc modification (Figure 4.10 B). Figure 4.10 B indicates that in general histone proteins possess a higher amount of O-GlcNAc during the S phase, and to a lesser degree at the beginning of the G2-phase and at G2/M transition. The level of O-GlcNAc on histones H2A, H2B and H3 remains quite high during cell cycle, but shows an increase during the S-phase. Similarly histone H1 has an elevated level of O-GlcNAc moieties during the S-phase and early G2-phase.

A total protein extract containing all proteins soluble at pH 7.4 was analyzed by Western blotting and immunodetection using the CTD110.6 antibody. The highest level of O-GlcNAcylation on this protein fraction was found during the G2-phase and at G2/M transition (Figure 4.11). This pattern was observed for protein polypeptides with a molecular masses of approximately 17 kDa, 26 kDa and 43 kDa as well as for some high molecular weight polypeptides that migrate at the top of the gel.
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Figure 4.1 Western blot on acid-extracted proteins from synchronized *N. tabacum* cv Xanthi cells using an antibody against histone H3 (A). Western blot analysis using the CTD110.6 antibody directed against O-GlcNAc, shows the cell cycle dependency of O-GlcNAcylation of the histones (B). Equal amounts of protein (2 µg) were loaded in each lane.

Figure 4.11 Western blot on total protein fraction extracted from synchronized *N. tabacum* cv Xanthi cells at pH 7.4 using an antibody against directed against O-GlcNAc (CTD110.6). Equal amounts of protein (4 µg) were loaded in each lane.
4.4 Discussion

In 2011, Schouppe et al. suggested that Nictaba is interacting with histone proteins through a specific binding of the lectin with the O-GlcNAc modification on the histones. This interaction was shown by pull-down assays using nuclear protein fractions from N. tabacum cv Xanthi cells and a recombinant His-tagged Nictaba, and was confirmed by lectin affinity chromatography of a commercial preparation from calf histones (Schouppe et al. 2011). O-GlcNAc modification is a well studied post-translational modification, especially in animal cells. However, in plants cells little research has been done concerning this carbohydrate modification. Here, we synchronized N. tabacum BY-2 suspension cells as well as N. tabacum cv Xanthi cells, to determine if plant histones are O-GlcNAc modified and if so, whether this modification is cell cycle-dependent.

Synchronized BY-2 cell cultures were obtained after treatment with aphidicolin, an inhibitor of the DNA polymerase α blocking cells at G1/S transition. Flow cytometric results allowed to determine when the cell culture entered the different phases of the cell cycle. Using flow cytometry, clearly defined sharp peaks were obtained, indicating a good degree of synchrony of the tobacco cells. Compared to previous reports in literature, the mitotic phase of the synchronized wild-type BY-2 cell culture occurred somewhat later (8 h after aphidicolin release compared to 6 h) (Swiatek et al. 2002; Gemperlova et al. 2009). Possibly, the aphidicolin removal was not totally efficient and the remaining chemical caused a prolonged S-phase.

Synchrony of the cell cycle was also assessed by analyzing the phosphorylation of histone H3 on Ser10. The phosphorylation of H3 Ser10 was previously shown to be initiated during the late G2-phase and is present until the end of mitosis and hence can be used as a mitotic marker (Houben et al. 2007). In the synchronized BY-2 culture H3S10ph modification was already observed during the G2-phase and the signal was decreasing during the M-phase. This discrepancy was also observed by Zhang et al. (2011) and could be due to the fact that a fraction of cells considered to be in the G2-phase already enter the mitotic phase. In addition, it should be mentioned that the antibody used directed against phosphorylation of Ser10 from histone H3 was only tested in animal cells (Zhang et al. 2011).

Synchronization of N. tabacum cv Xanthi cells was a more challenging task, but necessary, since N. tabacum cv BY-2 cells do not express Nictaba. In literature, there are only a few reports describing N. tabacum cv Xanthi cell synchronization (Nishinari and Syōno et al. 1986, Qin et al. 1996). This experiment has not been performed frequently, simply because synchronization of N. tabacum cv Xanthi cells is cumbersome due to the long cell cycle taking up to 25 hours. Therefore, N. tabacum BY-2 cells are the better alternative, due to its much shorter cell cycle and the higher synchronization
efficiency. *N. tabacum* cv Xanthi cells were synchronized using a similar protocol as for the BY-2 cells and a mitotic peak was detected 12 hours after drug removal. While analyzing the samples, it was clear that the level of synchronization obtained for the *N. tabacum* cv Xanthi cells was much lower compared to the BY-2 cells. Unfortunately, the flow cytometric analysis was not successful. The isolation of the nuclei was apparently insufficient although preliminary tests yielded good results. Since *N. tabacum* cv Xanthi cells show a lower degree of cell cycle synchrony, the results obtained with this experiment should be interpreted with care.

Every hour during cell cycle progression, samples were taken for analysis at RNA and protein level. To study gene transcription, semi-quantitative RT-PCR and quantitative real-time PCR were performed. Protein expression was examined using specific antibodies directed against the protein or the modification of interest.

Nictaba expression and its localization in the plant cell were investigated after cell cycle synchronization of wild-type *N. tabacum* cv. Xanthi cells and a transgenic line of *N. tabacum* BY-2 cells overexpressing the EGFP-Nictaba fusion protein under the control of the 35S promoter. Transcription of the Nictaba gene was shown to be cell cycle-dependent. The lectin is highly expressed in *N. tabacum* cv. Xanthi cells during the S- and G2-phase but expression decreases during the mitotic phase. The localization pattern for EGFP-Nictaba in the BY-2 cells appears to be unrelated to cell cycle progression since continuous overexpression of the EGFP-lectin yields fluorescence in the nucleus and cytoplasm of the cell at every time point of the cell cycle.

Histone H1 and H3 transcripts in *N. tabacum* cv. BY-2 cells were analyzed because of their known gene expression profile. In general, histone transcript quantity increases and decreases in parallel to DNA synthesis during the cell cycle. During the S-phase, the chromosomal DNA but also the chromatin structure need to be replicated (Meshi *et al.* 2000). Both RT-PCR and qPCR confirmed that the mRNA transcripts for H1 and H3 are indeed more abundant during the S-phase. Furthermore RT-PCR analysis also suggested a higher level of histone H1 transcripts compared to H3 transcripts. This result was confirmed by real-time PCR analyses that yielded up to 4 fold upregulation for H1 transcripts during the S-phase compared to two-fold up-regulation for H3 transcripts. Concomitantly, an increase in histone H3 protein synthesis was found after DNA synthesis had started. In the synchronized *N. tabacum* cv. Xanthi cell culture, the cell cycle-dependent expression of histone H3 deviated from this pattern, presumably because of the lower efficiency of synchrony of the cells.

In both *N. tabacum* cell types the enzyme OGT showed high transcript levels during the G2-phase and to a lesser extent during the M-phase. Unfortunately, qPCR results could not completely validate
these data because the results obtained for the samples collected during the G2-phase were not significantly different from the control. However, consistent with these findings, O-GlcNAcylation of tobacco histones was found during the G2/M transition and the M-phase. Furthermore, tobacco histones were also glycosylated during the S/G2 transition. Our results are in accordance with the study done by Zhang et al. 2011 on HeLa cells, since an increase of O-GlcNAc residues was observed as the cell cycle progresses through the G2- and M-phase, but glycosylation during the S/G2 transition phase was not shown. However, O-GlcNAcylation during the S/G2 transition was described by Sakabe et al. 2010 and Fong et al. 2011. All these studies were performed in human cells.

Little is known about O-GlcNAc modification of proteins in plants. Our results clearly showed that similar to animal histone proteins, also the plant histones are post-translationally modified with O-GlcNAc. Next to the acid-extracted histones, the analysis of a total protein fraction extracted at pH 7.4 also showed a high degree of O-GlcNAcylation of proteins during the G2-phase and during the G2/M transition. In this chapter, we detected for the first time the presence of O-GlcNAc on plant proteins, especially on histone proteins. Furthermore, cell cycle dependency of the O-GlcNAc modification on histone proteins was also demonstrated in plants. Because this histone modification takes place at the transition points of the cell cycle, it might be an important regulator of cell cycle progression.

The interaction between Nictaba and the core histones can only occur at those time points when lectin expression and O-GlcNAcylation of the histones occur simultaneously. Particularly this means that interaction could occur during the S/G2 transition and during the G2-phase, since at this time O-GlcNAcylation of histones reaches the highest level and Nictaba is expressed. O-GlcNAc modification of histones has recently been discovered as an important PTM that could be associated with the epigenetic regulation of transcription. It is possible that O-GlcNAcylation of histones recruits effector protein complexes influencing gene expression. Whether Nictaba is present within such a complex and whether the final result of this interaction will be activating or repressing chromatin transcription should be investigated in more detail. For instance, it has been demonstrated that acetylation is a PTM that is activating transcription, whereas SUMOylation represses transcription. However, methylation and ubiquitylation have variable effects, e.g. a single mark, H3K4me3, can recruit different proteins with both activating and repressing functions (Berger 2007). It is tempting to speculate that through the interaction with the core histones, Nictaba can directly or indirectly alter gene transcription.

In conclusion, the occurrence of O-GlcNAc on plant proteins was shown for the first time. In particular, the O-GlcNAc modification of plant histones was scrutinized and was also demonstrated
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to be cell cycle-dependent. The suggested interaction between Nictaba and O-GlcNAcylated histones presumably happens during S- and/or G2-phase, since during these phases both are simultaneously present within the nucleus.

4.5 Material and methods

4.5.1 Cell cultures

_N. tabacam_ cv ‘Bright Yellow-2’ (BY-2) cells were grown as indicated in Chapter 3, 3.5.1. 

_N. tabacam_ cv Xanthi cells were supplied by Dr Stéphane Bourque (Université de Bourgogne Plante – Microbe – Environnement, Dijon Cédex, France) and were grown in 250 ml erlenmeyers on an orbital shaker (25°C, 150 rpm, constant darkness). Cell suspensions were maintained in 100 ml medium containing 4.4 g/L Linsemaier-Skoog salts (Duchefa), 0.2 g/L glutamine and 30 g/L saccharose (adjusted to pH 5.6 with 1 M KOH). Before subculturing the cells, this medium was supplied with 100 µl of a 1000-fold concentrated vitamin solution (0.5 mg/L pyridoxine, 2 mg/L biotin, 0.1 mg/L thiamin, 5 mg/L nicotinic acid, 3 mg/L Ca\[^{2+}\] panthotenic acid and 2 mg/L glycine), folate solution (0.5 mg/L folic acid) and hormone solution (0.165 mg/L 2.4-D and 0.1 mg/L kinetin). Cells were subcultured weekly by transferring 8 ml dry cells into 100 ml fresh medium.

4.5.2 Stable transformation of Bright Yellow – 2 cells and confocal microscopic analyses

BY-2 cells were transformed as indicated in Chapter 3, 3.5.6. Confocal microscopic analysis was performed as depicted in Chapter 3, 3.5.7.

4.5.3 Cell cycle synchronization and monitoring of synchrony

The synchronization protocol was based on the method of Kumagai-Sano _et al_. (2007). The stationary BY-2 cell culture was diluted 10:100 in fresh medium, supplemented with 4.4 mg/L aphidicolin (Sigma-Aldrich). After 24 h of culture, the aphidicolin was removed by extensive washing and resuspension of the cells in fresh medium. Every hour, samples were taken from the synchronized culture for a period of 14 hours. The cell cycle progression was monitored by determination of the mitotic index. Therefore, 0.5 ml cells were sampled every hour, fixed in a solution of ethanol/acetic acid (3:1 (v/v)) and washed with MS. After DNA staining with 4’,6-diamidino-2-phenylindole (DAPI), cells were again washed with MS, observed under an epifluorescence microscope Nikon Ti (Nikon Belux) and dividing cells were counted. Three hundred cells were counted for each timepoint. Stages from prophase to telophase were considered as the mitotic phase. Eight-milliliter samples were collected for analysis of the DNA content using flow cytometry. To release the cell nuclei, Galbraith’s buffer (45 mM MgCl\(_2\), 30 mM Na-citrate, 20 mM MOPS and 1 g/L Triton X-100, pH 7.0) was added to
a sample of frozen cell pellet, which was carefully chopped with a razor blade and filtered (40 µm mesh) prior to the addition of propidium iodide (0.5 mg/ml). The ploidy of the nuclei was determined using the flow cytometer (Epics Altra; Beckman).

4.5.4 Analytical techniques

Eight-milliliter samples were crushed in liquid nitrogen, lysed using glass beads and protein was extracted in PBS (137 mM NaCl, 8 mM Na$_2$HPO$_4$, 2H$_2$O, 3 mM KCl, 1.5 mM KH$_2$PO$_4$). The supernatant was used for analyzing the protein fraction soluble at pH 7.4. Subsequently, the pellet that remained after the first extraction was used for a second extraction using 0.2 M H$_2$SO$_4$ to collect the acid soluble proteins. These extracts were used for Western blot analysis. After separation of the proteins by SDS-PAGE in 15% acrylamide gels, immunoblot analysis was performed. The membrane was blocked with Tris-buffered saline (TBS; 10 mM Tris-Cl, 150 mM NaCl, 0.1% (v/v) Triton X-100, pH 7.6) containing 5% (w/v) non-fat milk powder, for one hour. Primary antibodies used were a polyclonal rabbit antibody directed against Nictaba (1:80) (Vandenborre et al. 2009), a polyclonal rabbit antibody against histone H3 (diluted 1:30000, Abcam, Cambridge, United Kingdom), a monoclonal mouse antibody against O-GlcNAc CTD 110.6 (1:1000, Covance, California, USA) and a monoclonal mouse antibody against Phospho-Histone H3pSer10 (1:1000, ThermoFisher Scientific, Rockford, USA). After incubation with the primary antibody for 60 minutes, the membrane was incubated with TBS supplemented with a horseradish peroxidase-coupled secondary antibody, goat anti-rabbit (diluted 1:300, Sigma-Aldrich) or rabbit anti-mouse IgG (diluted 1:1000, Dako Cytomation) depending on the primary antibody, again for 60 minutes. Immunodetection was achieved by adding enhanced chemiluminescence detection substrate (Clarity™Western ECL Substrate, Bio-Rad, Hertfordshire, UK). Visualization was done using the ChemiDoc MP imaging system (Bio-Rad). The intensity of the bands on the blots was measured using the gel analysis tool from Fiji (http://fiji.sc/Fiji).

4.5.5 RT-PCR analysis

RNA was extracted from eight-milliliter samples and 1 µg was transcribed into cDNA using the First Strand cDNA Synthesis RT-PCR kit (Invitrogen, Carlsbad, California, USA). The presence of cDNA encoding histone H1, histone H3, O-GlcNAc transferase and Nictaba was analyzed by RT-PCR. The primers used to amplify the cDNA for each of the targets under study are shown in Appendix A. Cycling parameters were as follows: 2 min 94°C, 30x (15 s 94°C, 30 s 55 °C, 1 min 72°C), 5 min 72°C. The intensity of the bands on the blots was measured using the gel analysis tool from Fiji (http://fiji.sc/Fiji).
4.5.6 qPCR analysis

The SensiMixTMSYBR No-ROX Kit (BIOLINE, London, UK) was used to perform the qRT-PCR reactions. In each reaction, 10 µl of 2 x SensiMix, 1 µl cDNA (20 ng/µl), 1 µl of 10 µM from each primer and 7 µl water were mixed in a total volume of 20 µl. All reactions were performed in the Rotor-Gene 3000 (Corbett Life Science, Qiagen, Venlo, The Netherlands) using Rotor Discs (Qiagen), and the results were analyzed using the Rotor-Gene 6 software. The thermal profile consisted of 10 min at 95 °C as a pre-denaturation step, 45 cycles of 25 s at 96 °C, 25 s at 60 °C, and 20 s at 72 °C. To test the amplicon specificity, a melting curve was generated by increasing the temperature from 72 °C to 95 °C. The relative expression level of the target genes under stress conditions were compared with the control by using the REST 384 software (Corbett Research, (Pfaffl et al. 2002)). This software allowed determining the statistical significance of the results and compared the relative expression between a sample and a control group. REST 384 software analyses data for significant differences using ANOVA approximate tests (Pfaffl et al. 2002). Primers for qRT-PCR were designed by Primer3Plus software: http://primer3plus.com/cgi-bin/dev/primer3plus.cgi to amplify <150 bp amplicons (Appendix A).
Chapter 5

Study of the *in vitro* and *in vivo* interaction between Nictaba and the core histone proteins
Chapter 5: Study of the *in vitro* and *in vivo* interaction between Nictaba and the core histone proteins

*Manuscript in preparation*

Chapter 5: Study of the *in vitro* and *in vivo* interaction between Nictaba and the core histone proteins

5.1 Abstract

Nictaba is a nucleocytoplasmic lectin which is expressed in tobacco (*N. tabacum*) leaves treated with jasmonates or subjected to insect herbivory. Consequently, it is believed that Nictaba acts as a signaling protein involved in the stress physiology of the plant. In previous research, a nuclear proteomics approach was followed to search for binding partners of Nictaba. Using a pull-down assay and lectin affinity chromatography with extracts from *N. tabacum* cv Xanthi nuclei, histones were identified as primary interaction partners for Nictaba. This interaction was confirmed *in vitro* by affinity chromatography using purified calf thymus histone proteins on a Nictaba column.

In this chapter, the interaction between Nictaba and tobacco histones is scrutinized both *in vitro* and *in vivo*. To this end, a protocol was developed to purify histones from tobacco BY-2 suspension cells. Using lectin chromatography and far Western blot, the interaction between tobacco histones and Nictaba was confirmed. Furthermore, co-localization studies showed that both binding partners reside in the same cellular compartment, the nucleus. To assess the interaction *in vivo*, bimolecular fluorescence complementation (BiFC) and fluorescence resonance energy transfer (FRET) experiments were performed. The results of both microscopic techniques could not irrefutably confirm the interaction between Nictaba and histones.

5.2 Introduction

Protein-protein interactions (PPI) are fundamental to the normal functioning of every biological system. They are involved in virtually every cellular process, such as establishing specificity between enzymes and substrates in signal transduction pathways, DNA replication, cell cycle control, stress responses and building up the cytoskeleton. Knowing a protein’s interaction partner(s) is essential to understand the protein’s function. Therefore, in recent years, an increasing number of protein-protein interaction studies have been performed both in mammals and plant systems, although the generation of interactome maps for plants is lagging behind (Lalonde *et al.* 2008; Braun *et al.* 2013).

PPI differ from each other by varying kinetics and thermodynamics. According to the nature of the interaction either, stable or dynamic, proteins associate with a high or low affinity. These properties must be considered when an appropriate PPI detection method is selected (Lalonde *et al.* 2008).

Several techniques are commonly used to discover new interactions both by heterologous expression in yeast or purification from plants. Yeast two-hybrid (Y2H) has been extensively used in animal and plant interaction experiments. It is the only available high-throughput assay and is often used for screening DNA libraries for new interactors, although it is only suitable for the detection of nuclear protein interactions. Furthermore, Y2H suffers from a high false positive rate and only binary
interactions can be examined (Zhang et al. 2010; Braun et al. 2013). Based upon Y2H, the first proteome-wide interaction map for A. thaliana was published in 2011 (Arabidopsis Interactome Mapping Consortium, 2011). The Y2H protocol has also been improved for use in Arabidopsis protoplasts to avoid missing undetectable interactions in heterologous systems (Ehlert et al. 2006).

Protein complexes can also be extracted from plants using an affinity purification combined with MS (AP-MS). Co-immunoprecipitation is interesting because the endogenous protein is examined, it gives the most convincing evidence that proteins physically interact in vivo, although a specific antibody is required (Miernyk and Thelen 2008). Tandem affinity purification (TAP) is a very powerful technique but the stringent conditions limit identification of weak or low abundance PPIs. TAP followed by MS-based protein identification is one of the most frequently used techniques to study interaction networks in planta. Using this AP-MS platform, Van Leene and colleagues studied cell cycle-related protein complexes in Arabidopsis suspension cell cultures and identified 28 new protein association and confirmed 14 previously described interactions (Van Leene et al. 2007). One-step tag-based affinity purification is less stringent but suffers from a high false-positive rate. In planta, a GFP-tag is frequently used, combining interaction and localization studies (Braun et al. 2013). Identification of transient interactions and/or interactions characterized by high dissociation constants is typically done by chemical cross-linking agents, e.g. formaldehyde, which stabilizes the PPI. Since artificial associations are also stabilized, a more profound validation becomes necessary (Miernyk and Thelen 2008).

In Y2H, proteins are often overexpressed, hence altering the relative concentration of putative interactors compared to the in vivo situation. Heterologous expression can also exclude competing interactions or introduce novel ones. AP-MS experiments can assemble proteins from different cellular compartments which is not an accurate reproduction of the natural situation. Therefore, PPIs detected using these methods are considered as potential interactors and must be validated preferably in planta using an independent technique (Lalonde et al. 2008; Morsy et al. 2008). Protein-fragment complementation assays (PCAs) like bimolecular fluorescent complementation (BiFC) and split luciferase but also co-immunoprecipitation and fluorescence resonance energy transfer (FRET) are generally used PPI validation techniques (Braun et al. 2013).

In PCAs a reporter protein is split into two fragments that are fused to the proteins to be tested for interaction. In contrast to Y2H, PCAs allow detection of PPIs in diverse subcellular compartments. The split ubiquitin assay is for example especially used for analyzing interaction of membrane proteins (Morsy et al. 2008; Braun et al. 2013).
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BiFC is based on the association of two non-fluorescent fragments of GFP variants, reconstituting its fluorescent capacity (Hu \textit{et al.} 2002). This reaction is quasi-reversible, which makes BiFC the perfect technique for detection of weak or transient interactions, although this can also result in false positives. BiFC is applicable in various cellular compartments and allows detection of the localization of the interaction complex (Bhat \textit{et al.} 2006; Lalonde \textit{et al.} 2008; Zhang \textit{et al.} 2010, Braun \textit{et al.} 2013). In 2004, Bracha-Drori \textit{et al.} and Walter \textit{et al.} reported the usage of BiFC in plants. Since then, the system has been routinely used for studying protein complexes in plant cells (Bracha-Drori \textit{et al.} 2004; Walter \textit{et al.} 2004; Citovsky \textit{et al.} 2006; Marion \textit{et al.} 2008; Citovsky \textit{et al.} 2008).

Alternatively, the split luciferase assay is used to report protein interactions. In plants, this assay was first described in Arabidopsis protoplasts using the Renilla luciferase (Fujikawa and Kato, 2007) and later in \textit{N. benthamiana} leaves and Arabidopsis protoplasts using the firefly luciferase (Chen \textit{et al.} 2008). The split luciferase assay does not suffer from background light and can detect protein dissociation, it is thought to be the most sensitive and dynamic PPI detection method, although light emission is rather dim, so detection requires a very sensitive charge-coupled device camera (Morsy \textit{et al.} 2008).

FRET is characterized by the efficiency of the energy transfer \( E \) between a donor and an acceptor fluorophore both attached to two putative interacting proteins. \( E \) is defined as the fraction of the photons absorbed by the donor and transferred to the acceptor and is function of the distance between the two fluorophores. FRET can occur in the range of 1-10 nm, which is a relevant distance for two interacting proteins (Vogel \textit{et al.} 2006; Lalonde \textit{et al.} 2008). At present, the most widely used donor-acceptor pair is CFP/YFP, although poor spectral overlap does not make it the ideal choice. EGFP/mRFP1 would be a superior FRET partnership, using longer, less phototoxic excitation light, if mRFP1 displayed a stronger brightness (Campbell \textit{et al.} 2002; Dixit \textit{et al.} 2006). This donor-acceptor FRET pair was successfully used by Mas \textit{et al.} 2000 to analyze the interaction between Arabidopsis phytoreceptors PHY-B and CRY-2 (Mas \textit{et al.} 2000). FRET can be determined using different procedures of which donor fluorescence recovery after acceptor photobleaching is most frequently used because of lack of bleed-through and less sensitivity to artifacts caused by unequal expression levels. The most advanced way to determine FRET is measuring the fluorescence lifetime of the donor fluorophore, which decreases faster in close proximity to an acceptor fluorophore (Bhat \textit{et al.} 2006).

Besides experimental approaches, computational analysis of previously published interactions combined with connections between interologs, orthologous proteins known to interact in model species, are used to build an interactome network (Uhrig 2006; Zhang \textit{et al.} 2010; Fukao 2012).
Geisler-Lee and colleagues created an interactome for Arabidopsis based on interologs present in yeasts, nematodes and humans (Geisler-Lee et al. 2007).

In 2011, Schouppe et al. detected the protein interaction between the tobacco lectin and the core histone proteins H2A, H2B and H4 using an AP-MS approach. Two independent approaches, lectin affinity chromatography and pull-down assay allowed to purify histones from tobacco cv Xanthi nuclei and protoplasts, and identified these proteins as interaction partners for Nictaba. Furthermore, the interaction between Nictaba and histones was confirmed by lectin affinity chromatography of a commercial histone preparation from calf thymus (Schouppe et al. 2011). In this chapter, the interaction between Nictaba and plant histones was investigated in more detail. Therefore, histones were purified from BY-2 cells and the interaction between Nictaba and these tobacco histones was examined in vitro using lectin affinity chromatography and far Western blot analysis. The interaction was further evaluated by several microscopy techniques including co-localization studies, BiFC and FRET analyses.

5.3 Results

5.3.1 Purification of tobacco histone proteins

In 2011, Schouppe et al. identified histones as interacting partners for Nictaba, this interaction was tested by biochemical approaches using histone proteins from calf thymus. Although histone proteins are known to be highly conserved proteins in eukaryotes, it was of great interest to confirm the interaction of the lectin with plant histone proteins. Therefore a protocol was developed to purify histone proteins from tobacco BY-2 cells. This protocol can be subdivided into three main fractionation/purification steps. First, the cell wall was removed enzymatically to obtain a protoplast solution. Second, these protoplasts were lysed and nuclei were collected using an iodixanol gradient. Finally, these nuclei were incubated under acidic conditions to acquire the histones. Every step of this protocol was monitored by microscopy (Figure 5.1). Figure 5.1 A shows the successful isolation of protoplasts and DAPI staining confirms the purification of tobacco nuclei (Figure 5.1 B).
Figure 5.1 Microscopical confirmation used to monitor the different steps of the tobacco nuclei purification protocol. Transmission microscopy image of BY-2 protoplasts (A). Nuclei extracted from BY-2 cells stained with DAPI (B-1) visualized under transmission light (B-2) and a composite image of these two images (B-3). Scale bars are 50 µm.

The acid-purified proteins extracted from the nuclei were subjected to different analytical techniques to verify if the procedure allowed purification or enrichment of tobacco histones. The protein sample was separated by SDS-PAGE and Coomassie stained (Figure 5.2 A). Based on the molecular masses (See Chapter 4 Table 4.1) the protein bands could correspond to the core histone proteins. In addition, Western blot analysis was performed to confirm the identity of these bands. Antibodies directed against histones H3 and H4 were used to prove the presence of these proteins in the purified sample (Figure 5.2 B and C). Furthermore, mass spectrometry of the acid-soluble proteins validated the occurrence of histone proteins H2A, H2B, H3 and H4. Histone H3 has three known sequence variants denoted as H3.1, H3.2 and H3.3. All these variants were present in the histone preparation. Because of their highly similar sequence, they are further referred to as histone H3. Since the interaction between Nictaba and histones is dependent on histones carrying an O-GlcNAc residue, the O-GlcNAcylation of these proteins was also evaluated. Western blotting with an antibody directed against O-GlcNAc (CTD 110.6) resulted in clear bands at the position corresponding
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to the histone proteins, indicating that the post-translational modification was preserved (Figure 5.2 D).

![Image of SDS-PAGE and Western blot analysis](image)

**Figure 5.2** SDS-PAGE (A) and Western blot (B-D) analysis of acid-soluble protein from tobacco BY-2 cells. The different proteins were identified based upon molecular mass and specific antibodies directed against histone H3 (B) and histone H4 (C). The purified histones were modified with O-GlcNAc, as confirmed by Western blot analysis using the O-GlcNAc-specific antibody CTD 110.6 (D). In lane A, 2 µg of protein was loaded, in lane B and C 1 µg and in lane D 2.5 µg.

5.3.2  *In vitro* interaction study between the tobacco lectin and tobacco histones

Two biochemical binding assays were performed to examine the interaction between Nictaba and the tobacco histone proteins.

5.3.2.1  *Far Western blot analysis*

Far Western blot is a commonly used approach to detect protein-protein interactions *in vitro*. This technique is derived from the standard Western blot method. Purified histone proteins (prey) were first separated by SDS-PAGE and transferred to a membrane. The membrane was blocked and probed with purified Nictaba (bait) protein. If the Nictaba-histone interaction takes place, the bait protein can be immunodetected on spots in the membrane where a prey protein is located. Figure 5.3 shows an ECL detection of histone proteins H2A, H2B, H3 and H4 using an antibody directed against Nictaba, suggesting that these histone proteins do interact with Nictaba.
5.3.2.2 **Lectin affinity chromatography**

Complex formation between Nictaba and the histones was also studied with the Nictaba-Sepharose 4B column. Lectin affinity chromatography was first performed using a total non-fractionated preparation of histone proteins from calf thymus (Figure 5.4 A). Proteins bound to the lectin column were eluted by adding a buffer with high pH. Subsequently, eluted proteins were analyzed by SDS-PAGE, Western blot and MS analyses (Figure 5.4 A). Previously, Schouppe *et al.* 2011 established elution of calf thymus histone proteins from the Nictaba column with 1 M N-acetyl-D-glucosamine, pointing out the specificity of this interaction.

After validation of the lectin affinity chromatography with calf histones, a preparation of tobacco histones was also chromatographed on the Nictaba-Sepharose 4B (Figure 5.4 B). Analyses of the elution fractions corresponding with the highest absorption by MS confirmed the identity of histones H2A, H2B, H3 and H4. Furthermore, Western blotting using the CTD110.6 antibody showed the O-GlcNAc modification of histones H2A, H2B and H3.
Figure 5.4 Interaction of histone proteins with Nictaba-Sepharose 4B column. A, left panel: Elution profile of the histone proteins from a commercial calf thymus preparation on Nictaba-Sepharose 4B column. A, right panel: The most concentrated fraction from the elution peak was analyzed by SDS-PAGE and Western blotting using antibodies directed against histone H3 and O-GlcNAc. B, left panel: Elution profile of the purified tobacco histone proteins on Nictaba-Sepharose 4B column. B, right panel: The most concentrated elution fraction was analyzed by SDS-PAGE and Western blotting using antibodies directed against histone H3S10ph and O-GlcNAc.
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5.3.3 Co-localization studies of Nictaba in the plant nucleus

5.3.3.1 Co-localization between EGFP-Nictaba and DAPI stain

To confirm the nuclear localization of Nictaba, *N. benthamiana* leaves were co-infiltrated with EGFP-Nictaba and DAPI. Free EGFP known to locate to the plant nucleus was used as a control. To quantify co-localization, both Pearson and Manders’ coefficients were calculated. The Pearson correlation coefficient is a metric that describes the goodness-of-fit for a linear regression between all pixel grey-values of two different channels. Manders’ coefficients M1 gives a good idea of the proportion of green signal coinciding with a signal in the blue channel over its total intensity, whereas M2 gives the proportion of blue signal coinciding with the green signal (Bolte and Cordelières *et al.* 2006). Because the leaf material has a complex three-dimensional structure with different cellular organization, containing different expression levels of fluorescent proteins and different stoichiometries of association, the image is cropped to match only that specific structure to make sure that the coefficients will reflect co-localization of a local structure accurately.

Figure 5.5 A summarizes the correlation between EGFP-Nictaba and DAPI localization patterns. All values were positive and much higher than 0 indicating co-localization, albeit not perfect (i.e not equal to 1). However, this partial co-localization is also reflected in the images for free EGFP which is known to be abundant in the nucleus. The Manders’ coefficient M1 value gives the amount of EGFP-Nictaba overlapping with the DAPI staining. Since EGFP-Nictaba is not exclusively expressed in the nucleus, but also locates for an important part in the cytoplasm, the M1 value was low (Figure 5.5 B). Nevertheless, the fraction of DAPI co-localizing with EGFP-Nictaba (M2) is high, confirming the localization of Nictaba within the nucleus.
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Figure 5.5 Co-localization between EGFP-Nictaba and DAPI. Pearson and Manders’ correlation coefficients were calculated for EGFP-Nictaba and compared with results for free EGFP. Error bars represent standard deviations (A). Overlay picture from transiently transformed \textit{N. benthamiana} leaves co-infiltrated with EGFP-Nictaba and DAPI (B). Differences in co-localization coefficients between EGFP-Nictaba and EGFP were statistically significant ($p < 0.05$).

5.3.3.2 Co-localization between EGFP-Nictaba and histones

Co-localization experiments can be used to confirm if two proteins are both expressed in the same cellular compartment, which is an obvious condition for these proteins to interact. In general both proteins are tagged with a different fluorescent marker. Expression of each of these proteins individually will give one fluorescent signal. In the case of co-localization of both fluorescent proteins a mixed signal for both fluorochromes will be visualized.

Construction and testing of vectors for co-localization experiments

For the co-localization experiments, the constructs as described in Chapter 3 were used, encoding a fusion product consisting of EGFP C- or N-terminally fused to the coding sequence of Nictaba. Similarly, the different histone proteins H2A, H2B and H4 were covalently coupled with RFP in both configurations. Individual expression of the different fusion proteins was tested by Agrobacterium-mediated leaf infiltration of \textit{N. benthamiana} leaves. During these experiments, the standard protocol for transient transformation was optimized. The optimal concentration of Agrobacterium was
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defined at OD$_{600}$ 0.1. The optimal timepoint for checking the fluorescence of the transformed epidermal cells was on day 2 and 3 after infiltration for EGFP constructs and day 2 for RFP constructs. According to these observations, confocal microscopy was performed on day 2 after Agrobacterium infiltration.

All constructs but one yielded an intense fluorescence signal in the cellular compartment where the fusion protein was expressed, in particular the nucleus and cytoplasm for Nictaba and the nucleus for histone proteins. Only the Nictaba construct containing the C-terminally fused EGFP sequence did not give any detectable signal as discussed in chapter 3. Therefore, in further experiments, the construct encoding the fusion protein Nictaba-EGFP was no longer considered.

**Co-localization studies**

All possible combinations between EGFP-Nictaba and both N- and C-terminally tagged histone proteins were infiltrated in *N. benthamiana* leaves and examined using confocal microscopy (Figure 5.6 B). The expression of Nictaba was confined to the nucleus and the cytoplasm. Especially around the nucleus, a strong lectin expression was observed, within the nucleus the expression for EGFP-Nictaba was less intense but still higher than in the vacuole of the plant cell (Figure 5.6 A-1 and C). The histone proteins located exclusively in the nucleus (Figure 5.6 A-2). Figure 5.6 A-3 displays an overlay image of the green and red channel which clearly indicates the co-localization of the two examined proteins in the nucleus.

The co-localization between EGFP-Nictaba and H2B-RFP is also presented graphically in Figure 5.6 C. Briefly a virtual line was drawn through the nucleus and a plot profile was calculated for every fluorophore. Such a plot profile shows the fluorescence intensity for each position on the line. Both signals (red and green) can be shown in one graph, as such this shows the fluorescence of the two signals at a specific section through the nucleus. Four different plot profiles from the nucleus were made and these results were averaged. The percentage overlap between the two fluorescent signals was calculated and amounts to almost 60%. The confocal fluorescent images, shown in figure 5.6 C also clearly show a remarkably higher fluorescence intensity of EGFP-Nictaba fusion protein at the nuclear rim compared to the centre of the nucleus, whereas the histone proteins were evenly distributed in the nucleus.

All combinations of EGFP-Nictaba and N- or C-terminally labeled histones were analyzed and yielded similar results (Figure 5.6 B, Appendix B). These experiments clearly show that Nictaba is co-localizing with the histone proteins H2A, H2B and H4 inside the nucleus.
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![Image](71x466 to 531x771)

**Figure 5.6** Co-localization of EGFP-Nictaba with the different histone proteins fused with RFP. Panel A shows the localization of EGFP-Nictaba (A-1), the localization of H2B-RFP (A-2) and the overlay image (A-3). The scale bar is 25 µm. These results were representative for every combination of EGFP-Nictaba and the RFP labeled histones tested (B). Visualization of co-localisation between EGFP-Nictaba and H2B-RFP, the fluorescence overlap between the two signals is coloured yellow (C).

5.3.4 *In vivo* interaction study between Nictaba and tobacco histone proteins

Previous experiments showed that histone proteins and Nictaba co-exist in the same cellular compartment, the nucleus. However, co-localization does not directly implicate a protein-protein interaction, although this association has been suggested after *in vitro* studies performed by Schouppe *et al.* 2011. Therefore two commonly used approaches, BiFC and FRET were performed to study the *in vivo* interaction between Nictaba and histones.

5.3.4.1 Bi-molecular fluorescence complementation

*Design of fusion proteins for BiFC analysis*

For BiFC analysis, potential interacting partners were attached to specific fragments of fluorescent proteins. Only if the partners bind or interact with each other, these fragments will be joined and will form a functional fluorescent protein. Gateway-compatible vectors designed by Gehl *et al.* (2009) were used for this experiment. Because of the steric constraints of the interacting partners, the
characteristics of the endogenous proteins had to be reproduced as closely as possible. Therefore, both C- or N-tagging of each protein under study had to be investigated. The C terminal part of SCF3A (CFP\textsuperscript{C} or SCYCE) was both N- and C-terminal coupled with Nictaba. The N terminal part of VENUS (VENUS\textsuperscript{N} or VYNE) was covalently coupled with the different interaction partners, histone H2A, H2B and H4, also in both configurations. Every combination between these fusion proteins was tested (Figure 5.7). Only when the putative interacting proteins are in close proximity and the fluorophore fragments are in the correct orientation, a functional fluorophore is reconstituted and fluorescence will be emitted.

\textbf{Figure 5.7} Overview of fusion proteins constructed for the BiFC assays. The putative interactors are covalently fused with fragments of a fluorophore (CFP\textsuperscript{C} and VENUS\textsuperscript{N}). Multiple combinations of fusion proteins were tested to check for fluorescence emission. The fragments of the fluorophores are shown in grey to indicate that they cannot emit fluorescence (B and C). However, when two fragments interact and form a functional protein a fluorescent signal will be detected (A and D).

When the different BiFC constructs were made, a slight adaptation from the Gateway cloning protocol was necessary, because the destination vectors were created with the same antibiotic resistance gene as the entry clones. As a consequence, homologous recombination could still occur, but selection of the correct expression clones was impossible. To circumvent this problem LR recombination was performed between the destination vector and only a fragment of the entry
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clone. Therefore, a fragment of the entry clones, containing the recombination sites was amplified by nested PCR and used for further recombination with the destination vectors.

*Interaction between Nictaba and histone proteins*

Once the BiFC constructs for each of the fusion proteins were created, they were transferred into *A. tumefaciens* C58C1 pMP90 by tri-parental mating. Every possible combination of Agrobacterium strains containing plasmids expressing different fusion proteins, was infiltrated in leaf cells of *N. benthamiana*. Two days post-infection, fluorescence was monitored using confocal laser scanning microscopy. Only when Nictaba and the histones physically associated, a bi-molecular complex containing CFP<sup>C</sup> and VENUS<sup>N</sup> was formed and emission of fluorescence could be recorded. As shown in Table 5.1, almost half of the tested combinations resulted in emission of fluorescent light upon excitation suggesting that interaction between the proteins occurred, resulting in the formation of the active fluorophore (Figure 5.8, Appendix C).

![Figure 5.8](image)

**Figure 5.8** Confocal microscopy images of transiently transformed *N. benthamiana* leaves expressing Nictaba coupled with CFP<sup>C</sup> and histone protein H4 coupled with VENUS<sup>N</sup>. Interaction of these proteins gives an emission of green fluorescent light upon excitation (A). The red dots show the autofluorescence of the chloroplasts (B). The transmission image shows the surface of the leaves with the typical epidermis cells (C). In the right panel, all the images were merged and the complete picture is shown (D). Scale bar is 25 µm.
Table 5.1 Overview of different combinations of interaction partners tested for BiFC assays.

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<th>INTERACTION PARTNERS</th>
<th>BIFC RESULT</th>
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<tr>
<td>SCYCE-NICTABA</td>
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<tr>
<td>VYNE-H2A</td>
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<tr>
<td>H2A-VYNE</td>
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<tr>
<td>VYNE-H2B</td>
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<td>VYNE-H4</td>
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<td>H4-VYNE</td>
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<td>H4-VYNE</td>
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Table 5.1 gives an overview of all tested combinations and the corresponding results. According to these results, almost all histone VYNE fusion proteins, except the C-terminally tagged histone H2A, interacted with the SCYCE-Nictaba fusion protein. None of the interactions tested with the C-terminally tagged Nictaba yielded fluorescence emission upon excitation.

Control experiments for BiFC

Controls consisting of the Arabidopsis protein Cnx6, part of the molybdopterin synthase complex, were covalently fused with VYNE or SCYCE by Gehl et al. (2009). These protein fusions were used as negative controls since no evidence exists of an interaction between Cnx6 and Nictaba, or between Cnx6 and core histones proteins. For example, SCYCE-Nictaba fusion protein was tested for interaction with the VYNE-Cnx6 fusion protein. Every combination between Nictaba and VYNE-Cnx6 yielded a negative result. However, unexpectedly, all the combinations between the different histone proteins and SCYCE-Cnx6 resulted in emission of fluorescent light (data not shown).

To double-check these results, constructs were made expressing a Nictaba mutant incapable of binding sugar moieties (Schouppe et al. 2010). Since the interaction between Nictaba and histone proteins presumably occurs through binding of the lectin with the O-GlcNAc units present on the histone proteins, this mutant should no longer be capable to interact with the histones. Surprisingly, positive BiFC results were obtained when different combinations between this mutant Nictaba and the histone proteins were tested (Table 5.2), suggesting the presence of an additional binding mechanism.
Table 5.2 Overview of different combinations of interaction partners tested in BiFC assay using a mutant of Nictaba that lacks carbohydrate activity.

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<th>INTERACTION PARTNERS</th>
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<td>SCYCE-ΔCRB-NICTABA</td>
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<tr>
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5.3.4.2 Fluorescence resonance energy transfer

Since BiFC interaction studies could neither confirm nor enfeeble the interaction between Nictaba and histone proteins, another microscopy-based technique was used to study the in vivo interaction between both proteins. FRET describes the radiationless transfer of energy from a donor fluorophore to an adjacent acceptor fluorophore that has a significant spectral overlap and appropriate orientation. One of the techniques for measuring FRET is acceptor photobleaching, whereby the increase in donor fluorescence after complete acceptor photobleaching is a measure for the FRET efficiency. In our experiment, the EGFP-Nictaba fusion protein served as the donor fluorophore and the histones tagged with RFP as the fluorophore acceptors. Since EGFP and RFP have a spectral overlap, FRET efficiencies were calculated by measuring the increase in green fluorescence in the nucleus after the red fluorescence was completely bleached. Figure 5.9 shows the FRET efficiency calculated for EGFP-Nictaba combined with the histone H4-RFP fusion protein. After bleaching of the red fluorescent light (time point 5), the emission of the green fluorescent light increased with 3.5%. Table 4.3 gives an overview of the FRET efficiencies tested for all combinations between EGFP-Nictaba and the RFP-labeled histones. In addition, two Arabidopsis proteins fused with RFP were tested in a complete identical acceptor photobleaching experiment, combined with EGFP-Nictaba fusion proteins. These Arabidopsis proteins are not expected to interact with Nictaba and thus serve as a negative control in this experimental setup. The results show no difference in FRET efficiency calculated for Nictaba and histone proteins compared to Nictaba and the negative controls.
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**Figure 5.9** FRET efficiency calculated for the EGFP-Nictaba and the H4-RFP fusion proteins in an acceptor photobleaching experiment.

**Table 5.3** FRET efficiencies calculated for every combination between EGFP-Nictaba and the RFP-tagged histone proteins tested. Two unrelated and non-interacting proteins Arabidopsis FLA17 and ATS3-A fused to RFP were used as negative controls.

<table>
<thead>
<tr>
<th>Combination</th>
<th>FRET efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP-Nictaba</td>
<td></td>
</tr>
<tr>
<td>RFP-H2A</td>
<td>1.9 ± 0.97</td>
</tr>
<tr>
<td>H2A-RFP</td>
<td>5.4 ± 2.04</td>
</tr>
<tr>
<td>RFP-H2B</td>
<td>0.9 ± 1.34</td>
</tr>
<tr>
<td>H2B-RFP</td>
<td>0 ± 1.35</td>
</tr>
<tr>
<td>RFP-H4</td>
<td>1 ± 1.62</td>
</tr>
<tr>
<td>H4-RFP</td>
<td>3.5 ± 2.00</td>
</tr>
<tr>
<td>RFP-FLA17</td>
<td>1.7 ± 1.27</td>
</tr>
<tr>
<td>RFP-ATS3-A</td>
<td>3.3 ± 1.47</td>
</tr>
</tbody>
</table>

5.4  Discussion

Understanding protein interactions is extremely important to elucidate protein functions and networks. In an attempt to further scrutinize the association between Nictaba and the core histone proteins in vitro and in vivo, interaction studies were performed. For the in vitro binding studies, histone proteins were successfully purified from tobacco BY-2 cells. SDS-PAGE, Western blotting and MS analysis confirmed the occurrence of histones in the purified protein samples. In addition, O-GlcNAc modification was corroborated by Western blotting, using an antibody (CTD 110.6)
specifically directed against O-GlcNAc. The purified tobacco histones were used in a biochemical approach to investigate the interaction with Nictaba. Far Western blot analyses confirmed the interaction between the tobacco lectin and histones H2A, H2B, H3 and H4. Lectin affinity chromatography using a Nictaba-Sepharose 4B column with both histone proteins from calf thymus and tobacco histone proteins again endorsed their interaction. Previously, the interaction between Nictaba and calf histones was proven to be GlcNAc-dependent (Schouppe et al. 2011), therefore the eluted histone proteins were inspected for O-GlcNAcylation. All eluted tobacco histones contained the O-GlcNAc modification whereas in calf histones this modification was mostly confined to H2A, H2B and H3. These in vitro interaction studies give evidence for a direct interaction between the proteins of interest, although validation by an independent assay, preferably an in vivo study is essential.

Since a biochemical approach can bring proteins together that would never meet under physiological conditions, in planta co-localization studies were performed to validate that both proteins reside in the same cellular compartment. First, nuclear localization of Nictaba was judged by co-localization with DAPI, a fluorescent stain which binds strongly with A-T regions in DNA. Free EGFP, which can diffuse freely into the nucleus, was used as a positive control. Transient expression analysis was performed in tobacco leaves infiltrated with constructs expressing EGFP-Nictaba and EGFP. Expression of the proteins was analyzed by confocal microscopy, and co-localization was investigated. For both the free EGFP and the EGFP-Nictaba fusion protein correlation coefficients were calculated. The Pearson coefficient (PC) for EGFP-Nictaba was rather low, but the PC for free EGFP was not significantly different. Since PC values are highly dependent on noise, variations in fluorescence intensities or heterogeneous co-localization relationships throughout the sample also the Manders’ coefficients M1 and M2 were computed (Bolte and Cordelières 2006). The Manders coefficient M2 shows the amount of DAPI signal overlapping with the signal generated by the fusion protein EGFP-Nictaba signal and indicated co-localization with Nictaba in the nucleus.

Furthermore, co-localization between Nictaba and the histone proteins was scrutinized. Constructs expressing RFP fused to the histone proteins, in both the N- and C-terminal configuration, were transiently co-expressed with a construct encoding the EGFP-Nictaba fusion protein in the epidermis cells of N. benthamiana leaves. All possible combinations between Nictaba and histone constructs were tested and confocal microscopical analysis demonstrated the co-expression of the fusion proteins within the nucleus. A virtual cross-section of the nucleus showed the combined fluorescent signal of the proteins labelled with EGFP and RFP. Histone proteins are evenly distributed throughout the nucleus, whereas Nictaba levels are especially high at the nuclear rim.
Proteins residing in the same cellular compartment does not necessarily imply a direct physical interaction. Therefore, two independent microscopical analyses, BiFC and FRET, were performed. The interpretation of a co-localization experiment is limited by the maximum resolution of the used microscopical settings. For example, if the resolution is 200 nm a pixel displaying a double fluorescent signal is comparable with a sphere which still can contain 140,000 densely packed GFP-sized proteins. In contrast, a BiFC- or FRET-signal is comparable to a sphere containing about 100 GFP-molecules. Therefore, BiFC and FRET analyses can strengthen the evidence for protein interaction, but on their own they are insufficient to prove interaction (Vogel et al. 2006).

BiFC experiments were performed using histone fusion proteins, C- and N-terminally tagged with CFP and Nictaba fusion proteins coupled with VENUS, also in both configurations. All possible combinations were tested and only if putative interacting proteins came in close proximity, less than 10 nm, a functional fluorophore could be reconstituted and green fluorescent light could be observed upon laser light excitation. Almost half of the tested protein pairs resulted in a positive BiFC result. Negative results are most likely caused by sterical hindrance. These findings stress the importance of checking all possible protein fusions for both the N- and C-terminal ends, in an attempt to reproduce the characteristics of the endogenous protein as closely as possible (Bhat et al. 2006; Kerppola 2008; Kerppola et al. 2009). BiFC is based on the association of fragments of fluorescent proteins, which stabilizes protein interactions, and promotes the detection of weak and transient interactions. Because of this high sensitivity, it is necessary to add sufficient controls to a BiFC experiment (Kerppola 2008). First, control experiments were performed employing fusion proteins, VYNE-Cnx6 and SCYCE-Cnx6, which are not expected to interact with the proteins under study. BiFC assays in which the Cnx6 constructs were used in combination with the Nictaba constructs were negative, but in combination with constructs for the histone proteins the results turned out to be positive. No records of interaction between Cnx6 and histones have been reported in literature. To rule out the possibility of a true interaction, an additional control experiment was set up. Therefore, a construct encoding a mutant Nictaba protein, lacking carbohydrate binding activity and thus presumably incapable of interacting with histones, was used. In case a mutation changes the interaction interface, this will result in a significant change in the efficiency of the BiFC complex formation, and will suggest a specific interaction (Kerppola 2008; Kerppola et al. 2009). Unexpectedly, the construct encoding the Nictaba mutant yielded more positive BiFC results compared with the construct encoding the native Nictaba sequence. Possibly, this is due to the use of a constitutive promoter causing a great abundance of the proteins under study, particularly for the histones. Overexpression may favor interactions by chance rather than select for biologically relevant
interactions. Moreover the BiFC complex association is very stable, and thus once a (false) interaction has occurred, it is irreversible (Bhat et al. 2006; Kerppola et al. 2009).

FRET microscopical analyses are less prone to have false positive results than BiFC, because complexes are at equilibrium and can again dissociate, but a higher level of protein expression is necessary to detect energy transfer (Kerppola 2008). An acceptor photobleaching FRET experiment was carried out using the EGFP/RFP as the donor-acceptor pair. No problems concerning the lower brightness of mRFP, as described by Campell et al. 2002, were encountered during the experiment, probably because of the high expression levels and the rather restricted volume of the nucleus. Moreover, fluorescence intensities from RFP histone fusions are higher in the nucleus compared to those from EGFP Nictaba. FRET efficiencies were calculated based on measurements of donor fluorescence in the presence and the absence of acceptors. The obtained values were low, although it is difficult to compare FRET results between experiments reported in literature, because FRET standards, with known FRET efficiencies, have not been defined (Vogel et al. 2006). Although proteins not expected to interact with Nictaba delivered comparable results in the FRET analyses, this result does not imply that Nictaba and histones do not physically interact. It is for example possible that their dipoles are aligned perpendicular to each other and cancel each other out (Vogel et al. 2006) or labeling of the proteins of interest can put the fluorescent proteins too far away from each other to allow FRET, even if the proteins actually interact (Dixit et al. 2006). Since the FRET donor, EGFP-Nictaba is expressed at low levels in the nucleus, the FRET signal may appear relatively weak. Furthermore, endogenous proteins will compete for FRET partners, reducing the apparent FRET efficiencies (Dixit et al. 2006).

Ideally, to perform BiFC and FRET studies some general considerations should be taken into account. First, all possible pairwise combinations should be tested, second expression levels should mimic endogenous levels and third expression should take place in null mutations to avoid interference of untagged endogenous proteins (Bhat et al. 2006). In most cases, it is cumbersome to meet all these conditions, but in future experiments it should be feasible to replace the strong 35S promoter by a weaker promoter to overcome the problem of nonspecific BiFC (Citovsky et al. 2008; Caplan et al. 2008).

In conclusion, in vitro assays like far Western blots and lectin affinity chromatography confirmed the interaction between Nictaba and the histone proteins. Furthermore, co-localization studies corroborated the occurrence of both proteins within the nucleus. Both FRET and BiFC determine the close vicinity of two fluorophore-tagged fusion proteins in vivo, but did not give very conclusive
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results. FRET experiments could be expanded to FLIM, which is the most sophisticated technique but also a technically very demanding way to determine FRET (Bhat et al. 2006).

5.5 Materials and methods

5.5.1 Plant material

*N. benthamiana* and *N. tabacum cv ‘Bright Yellow-2’* (BY-2) cells were grown as described in Chapter 3, 3.5.1.

5.5.2 Preparation of tobacco histone proteins

4-day-old BY-2 cells were harvested using vacuum filtration and incubated at 37°C in an enzyme solution (2% cellulose RS (Duchefa), 1% macerozyme (Yakult, Brussels, Belgium), 0.1% pectolyase (Duchefa), 0.4 M mannitol (VWR, International, West Chester, PA USA), 40 mM CaCl$_2$ (VWR), 10 mM MES (Sigma-Aldrich), pH 5.5) for three hours with gentle shaking. The protoplast suspension was filtered through a 100 µm nylon cloth using wash buffer (10 mM MES, 0.4 M mannitol, pH 6.7), centrifuged for 5 min at 200 g and washed with wash buffer. Protoplasts were dissolved in 3 ml 20% Ficoll-400 (Sigma-Aldrich) in a 15 ml glass tube. On top of this layer 10% Ficoll-400 (Sigma-Aldrich) and wash buffer (0%) were added. After centrifugation for 30 min at 160 g at 4°C, purified protoplasts were recovered from the 10%-0% interphase. Subsequently, BY-2 protoplasts were suspended in ice-cold nuclei isolation (NIB) buffer (20 mM KCl (VWR), 20 mM HEPES (Duchefa), 0.6 % Triton X-100 (Thermo Fisher Scientific), 13.8% hexylene glycol (Sigma-Aldrich), 20 mM β-mercaptoethanol (Sigma-Aldrich), 50 µM spermine (Sigma-Aldrich), 125 µM spermidine (Sigma-Aldrich), 1mM phenylmethylsulfon fluoride (Roche Diagnostics GmbH, Mannheim, Germany), 2 µg/ml apoprotinine (Sigma-Aldrich), pH = 7.4) and passed 8 times through a 0.45 x 12 mm needle (Terumo, Somerset, USA) to completely lyse the cells. This solution was then filtered through a 31 µm mesh to remove unlysed protoplasts. To further purify the nuclei, the lysed protoplast solution was loaded on a 25%/36% iodixanol gradient (diluted in NIB buffer without Triton X-100, OptiPrep® Density Gradient Medium, Sigma-Aldrich) in a 15 ml glass tube. Gradients were centrifuged for 30 minutes at 4°C at 3,000 g and nuclei were isolated from the 25%/36% interphase. Purified nuclei were washed twice with NIB buffer without Triton X-100 to remove iodixanol and solvent remnants. After centrifugation for 30 min at 4°C at 3,000 g, the pellet containing nuclei was resuspended in a minimal amount of 0.2 M H$_2$SO$_4$ and vortexed. This acidic solution was incubated on a rotator (Stuart SB3, VWR) at 4°C overnight and thereupon centrifuged at 16,000 g for 10 min to remove nuclear debris. Undiluted TCA was added to the supernatant to a final concentration of 33%, to precipitate the histones, and this solution was incubated overnight on ice. Subsequently histones were collected
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by centrifugation at 16,000 g for 10 min at 4°C and washed twice with ice-cold acetone (VWR). The histone pellet was air dried at room temperature and finally dissolved in an appropriate volume of Milli Q water.

5.5.3 Western blot analysis

Purified tobacco histone proteins were analyzed by SDS-PAGE in 15 % acrylamide gels. Immunoblot analysis was performed by blocking the membrane first with Tris-buffered saline (TBS; 10 mM Tris-HCl, 150 mM NaCl, 0.1 % (v/v) Triton X-100, pH 7.6) containing 5 %(w/v) non-fat milk powder, followed by consecutive incubations in TBS supplemented with a primary rabbit antibody directed histone H4 (Abcam, diluted 1:12,500), a horseradish peroxidase-coupled goat anti-rabbit IgG (diluted 1:300, Sigma-Aldrich, Bornem, Belgium). Immunodetection was achieved by adding a sufficient amount of enhanced chemiluminescence detection substrate (Clarity™Western ECL Substrate, Bio-Rad). Visualization of the signals on the blot was done with the ChemiDoc MP imaging system (Bio-Rad). Western blot analysis for histone H3 and O-GlcNAc was performed as described in Chapter 4, 4.5.4.

5.5.4 Far Western blot analysis

Prior to Western blot analyses, SDS-PAGE was performed to separate the prey protein based on molecular mass. These separated proteins were subsequently blotted onto a PVDF membrane (Pall, New York, USA). The membrane was blocked with Tris-buffered saline (TBS; 10 mM Tris-HCl, 150 mM NaCl, 0.1% (v/v) Triton X-100, pH 7.6) containing 5% (w/v) BSA for one hour. Prior to immunodetection, the membrane is incubated with 10 ml of the bait protein Nictaba (1 µg/ml) for one hour. Then, a polyclonal rabbit antibody directed against Nictaba (1:80) was added for one hour, followed by a horseradish peroxidase-coupled goat anti-rabbit secondary antibody (1:25000, Sigma-Aldrich, Bornem, Belgium) also for one hour. Immunodetection was achieved by adding a sufficient amount of enhanced chemiluminescence detection substrate (Clarity™Western ECL Substrate, Bio-Rad). Visualization of the signals on the blot was done with the ChemiDoc MP imaging system (Bio-Rad).

5.5.5 Lectin affinity chromatography

Approximately 3 mg total Type II histone preparation from calf thymus (Sigma-Aldrich) or 1 mg purified tobacco histones were used for affinity chromatography using a Nictaba-Sepharose column, equilibrated with phosphate buffered saline (PBS, 137 mM NaCl, 8 mM Na₂HPO₄·2H₂O, 3 mM KCl, 1.5 mM KH₂PO₄, pH 7.2). The column was washed with 1 column volume of PBS and bound proteins.
were eluted with 20 mM 1.3 diaminopropane. Fractions were collected and analyzed by SDS-PAGE and Western blot.

### 5.5.6 Construction of plasmids for co-localization studies

The coding sequences of Nictaba (AF389848), histone H2A (AK228063), histone H2B (AK228050) and histone H4 (AK229241) (Riken, Ibaraki, Japan) were used to construct the different plasmids.

The Nictaba coding sequence was cloned into the vectors pK7WGF2.0 and pK7FWG2.0 (Karimi et al. 2002) using the Gateway™ Cloning technology of Invitrogen as described in Chapter 3, 3.4.2. Following the same strategy the coding sequences of histones H2A, H2B and H4 were cloned into the vectors pH7WGR2.0 and pH7RWG2.0 (Figure 5.11). The primers used for the first round of PCR for amplifying the attB-PCR products were evd 624/625 and evd 626/627 for H2A, evd 620/621 and 622/623 for H2B and evd 628/629 and evd 630/631 for H4. The attB sequences were completed in a second PCR using primers evd 2 and evd 4 (Appendix A).

**Figure 5.11** Vector maps of the Gateway vectors used for co-localization studies. Schematic representations of the vectors pH7WGR2.0 and pH7RWG2.0 for N- or C-terminal tagging of the histone sequences with RFP, respectively. LB and RB, T-DNA border sequences; p35S, CaMV 35S promoter; RFP, red fluorescent protein; att, Gateway recombination site; ccdB, toxin protein gene; T35S, 35S terminator; Kan, kanamycin resistance gene; Hyg, hygromycin resistance gene; Sm/SpR, streptomycin/spectinomycin resistance gene.

### 5.5.7 Construction of BiFC vectors

The BiFC constructs were generated by using GATEWAY-compatible BiFC binary destination vectors (Gehl et al. 2009). The entry clones used were the same as those for constructing the co-localization vectors. Since both sets of vectors have the same antibiotic resistance gene a different cloning strategy was used. After sequencing, the plasmid of the entry clones was used for a nested PCR using M13 primers and pfX DNA polymerase. This PCR was performed twice. For each PCR a dilution of the start material was used to reduce the amount of entry clone as much as possible. This PCR product
and the BiFC destination vectors were used for the final LR reactions using the Gateway® LR
Clonase™ Enzyme mix (Invitrogen) to obtain expression clones. The expression clones were
transformed via heat shock into *E. coli* cells. Transformants were selected on LB agar plates
containing kanamycin (50 µg/ml) and screened by PCR. Plasmid DNA was also checked by sequencing
using gene specific and/or vector specific primers (VYNE: evd 652 and evd 653; SCYCE: evd 654 and
ev 655) to confirm whether the protein and tag sequence are in the correct frame.

Following this strategy, the coding sequence of Nictaba was cloned into destination vectors pDEST-
SCYCE®gw and pDEST-gwSCYCE (Figure 5.12). The coding sequence of histones H2A, H2B and H4 was
recombined into destination vectors pDEST-VYNE®gw and pDEST-gwVYNE (Figure 5.12).

5.5.8  Agrobacterium-mediated transient expression in *N. benthamiana*

The procedure was followed as described in Chapter 3, 3.4.5 and 3.4.6. For every BiFC combination at
least three independent repetitions of the Agrobacterium infiltration were performed.

5.5.9  Confocal microscopy

Fluorescence microscopy was performed of the lower epidermis cells of leaf discs 2 days after
infiltration using a confocal laser scanning microscope, type Nikon A1R (Nikon). The specimens were
examined using the 40x Plan Apo ELWD objective lens (NA of 0.75). A 488 nm argon laser was used
for excitation of EGFP and chlorophyll autofluorescence. For EGFP, the emitted light was separated
by a beam splitter 560 nm long pass beam splitter and fluorescence was detected through a 525/50
nm and 700/75 nm bandpass for EGFP and autofluorescence, respectively. The 561 nm diode laser
was used to excite RFP. The emitted light first passed the beam splitting mirror 560 long pass and was then separated by a dichroic mirror 640 long pass and detected using 595/50 bandpass filter. For BiFC (Venus\(^\text{N}\)/S(CFP)3A\(^C\)) experiments confocal settings were similar as for EGFP detection.

For co-localization studies, the Pearson correlation coefficient and Manders’ coefficients were calculated on the cropped images using the JACoP plugin ([http://rsb.info.nih.gov/ij/plugins/track/jacop.html](http://rsb.info.nih.gov/ij/plugins/track/jacop.html)) for Fiji image analysis freeware ([http://fiji.sc](http://fiji.sc)) (Manders et al. 1993). To quantify co-localization, both Pearson and Manders’ coefficients were calculated. Because the leaf material has a complex three-dimensional structure with heterogeneous cellular organization, containing different expression levels of fluorescent proteins and different stoichiometries of association, the image was cropped (always to the same size) to match only that specific structure to make sure that the coefficients would reflect co-localization of a local structure accurately.

FRET efficiencies were calculated during an acceptor photobleaching experiment by measuring the fluorescence intensity after excitation of EGFP in the region of interest (60 x 60 pixel) before and after bleaching the RFP fluorescence. These results were compared with a reference with the same experimental set-up except bleaching of the RFP channel. Using the formula \([I_{i \text{ROI}}/I_{i \text{REF}}] \text{ROI} / [I_{i \text{REF}}] \text{REF}\), E was calculated. For every combination, this measurement was repeated at least 30 times.
Chapter 6

Discussion and Perspectives
Every living organism is constantly exposed to a continuously changing environment, which most of the time can be considered as ‘stress’. Due to their sedentary life style, plants cannot escape to limit this exposure to stress. Therefore, plants developed a series of defense mechanisms in an attempt to control the effect of varying external stimuli. Since Nictaba is induced by both jasmonates and herbivory, it is suggested that Nictaba has a role in the defense mechanism of the plant. In this final chapter, the physiological processes that precede and follow Nictaba expression will be discussed (Figure 6.1). Experiments for future research focusing on elucidating the physiological role of the tobacco lectin in the plant will be suggested.

6.1 Nictaba induction by stress

Nictaba is induced in the leaves of *N. tabacum* cv Samsun NN after herbivory by Lepidopteran pest insects and jasmonate treatment (Figure 6.1-1). Both chewing insects, such as *S. littoralis*, *M. sexta* and cell content feeders, such as *T. urtica*, have the capacity to induce Nictaba gene expression most probably through activation of the jasmonate pathway (Vandenborre *et al.* 2009a). Jasmonates are synthesized from the fatty acid α-linolenic acid (18:3) by the octadecanoid pathway. Upon wounding by insects, JA biosynthesis is initiated in the chloroplasts. The exact mechanism how plants are capable to perceive insect herbivory and trigger the plant immune system is not clear, but endogenous plant signals produced in response to tissue damage and herbivore-associated molecular patterns from insect secretions play an important role (Pieterse *et al.* 2012). After transfer into the cytosol, Jasmonate Resistant1 catalyzes the conjugation of JA with isoleucine into the bioactive form (+)-7-iso-JA-Ile, which binds to the F-box protein COI1, thereby mediating the interaction of COI1 with JAZ proteins. This leads to the assembly of a functional SCF<sup>COI1</sup> complex, an E3 ubiquitin ligase. Consequently, the JAZ proteins are targeted for 26S proteasome degradation. The JAZ proteins repress the JA pathway by blocking the basic helix-loop-helix (bHLH) transcription factor (TF) MYC2. In Arabidopsis it was shown that JAZ proteins act as part of a protein complex which recruits co-repressors of gene transcription, such as chromatin remodeling factors. Unfortunately, not all the components of this protein complex are known in *N. tabacum* (Shoji and Hashimoto 2011). Once JAZ proteins are degraded, MYC2 binds with JA-responsive elements present in promoter sequences of JA-responsive genes (Chini *et al.* 2009, Wasternack and Hause 2013). Several JA-responsive elements such as JASE1ATOPR1, G-boxes and T/GBOXATPIN have been identified in *silico* in the Nictaba promoter sequence (Chapter 2). In order to delimit the JA-responsive regions in the Nictaba promoter, GUS activity could be analyzed in transgenic tobacco plants, transformed with promoter fragments of different lengths.
Chapter 6: Discussion and perspectives

MYC2 can activate the transcription of early JA-responsive genes. Since Nictaba is a late response gene, it is unlikely that MYC2 activates transcription of Nictaba. Several other transcription factors belonging to the bHLH and R2R3 MYB TF family and TFs involved in other hormonal signaling pathways are also targeted by JAZ proteins (Pauwels and Goossens 2011). Possibly, Nictaba is activated by one of these transcription factors. However, MYC2 was also shown to activate proteins associated with wound response and defense against insect herbivores, which correlates with Nictaba expression (Pieterse et al. 2012). Possibly, an extra step is involved and Nictaba is not a primary but a secondary JA-responsive gene, which can explain the late response of Nictaba expression. MYC2 might activate TFs which are synthesized de novo and in turn bind with the G-boxes in the promoter sequence of Nictaba. Such a G-box sequence can be bound by G-box-binding factor (GBF)-type of basic leucine zipper proteins or bHLH transcription factors synthesized in response to JA (Pauw and Memelink 2005).

6.2 Nictaba expression during plant development

In Chapter 2, the jasmonate induced expression of Nictaba in different plant tissues and developmental stages was followed by promoter-GUS experiments using transformed Arabidopsis and tobacco plants. Despite some similarities such as Nictaba expression in the leaves, also important differences were found between the two plant systems, such as the absence of GUS staining in the apical meristem in tobacco in contrast with Arabidopsis and completely different staining patterns in the hypocotyl. Interestingly, these experiments also demonstrated for the first time the expression of Nictaba in the roots of tobacco plants, which was confirmed by Western blot analyses and ELISA. Furthermore, cold stress induced Nictaba expression in the roots but not in the leaves. How Nictaba expression in the roots is correlated with cold stress and if this has an effect on the physiology of the plant remains unknown. Whether this cold induced Nictaba expression is beneficial for the plant, could be tested for example by comparing the plant performance after cold treatment of wild-type N. attenuata plants, which lacks Nictaba expression, and N. attenuata plants ectopically expressing Nictaba.

During plant development Nictaba is highly expressed in the young developmental stages of the plant. The presence of Nictaba in the different plant tissues gradually decreases as the plant gets older. In addition, there seems to be a switch from expression in the youngest leaves in the beginning of plant development towards expression in the developed leaves as the plant gets older. Nictaba expression might be more important in the young tissues during seedling growth since these tissues are more vulnerable to stress conditions. The switch to older leaves in adult plants might be linked with leaf ageing.
After jasmonate treatment or insect herbivory, which is known to activate the jasmonate pathway, Nictaba expression is triggered. JA interacts with the COI1 receptor of the SCF complex and thereby recruits JAZ proteins which are subjected to ubiquitinylation and subsequent degradation by the 26S proteasome. Subsequently, transcription factors are unblocked and can activate transcription of Nictaba (1). mRNAs encoding Nictaba are translocated into the cytoplasm where they are translated on free polysomes (2) (A). After a certain amount of time and in the same cell, Nictaba is partly transported into the nucleus through the NPCs (3) where it can interact with core histones proteins, presumably to regulate gene transcription (4) (B). For more details we refer to the text.

6.3 Nictaba is expressed in the cytoplasm of the cell

After transcription, Nictaba mRNA is translated into protein on free polysomes in the cytoplasm (Figure 6.1-2). In Chapter 3, the cytoplasmic expression of Nictaba was confirmed by co-localization studies with an ABC-transporter with a known cytoplasmic localization. Since the resolution of the confocal microscope is not high enough to clearly distinguish between the cytoplasm and the cellular membrane, tobacco BY-2 protoplasts ectopically expressing the EGFP-Nictaba fusion protein were
treated with a nonionic detergent. This experiment corroborated that Nictaba is not associated with the cellular membrane.

At present, little is known about the physiological importance of Nictaba within the cytoplasm. Since Nictaba was shown to possess entomotoxic activity on larvae of Lepidopteran pest insects (Vandeborre et al. 2010), it is possibly a product of the direct defense reaction of the tobacco plant which produces molecules that act as toxins or feeding deterrents. To this end, (nuclear) localization is probably of minor importance.

Although Nictaba possesses a NLS, a large part of the lectin is found in the cytoplasm of the cell. Possibly, there is a cytoplasmic interaction partner serving as a cytoplasmic retention factor or masking the nuclear target sequence. Also phosphorylation can interfere with the interaction between the NLS and the importin and as such can interfere with nuclear import (Jans et al. 2000).

In 2011, Schouppe et al. initiated a search for interacting proteins in the cytoplasm by using an unfractionated protoplast extract in a pull-down assay. Besides a putative heat shock protein PS1, the majority of the identified proteins localized to the nucleus (Schouppe et al. 2011). In future work, a pull-down assay can be performed using a fraction of cytoplasmic proteins which is free of contaminations originating from cellular organelles or the cytoplasmic membrane. Such a fraction can be obtained by lysis of the cell followed by a centrifugation step (NE-Per Nuclear and cytoplasmic extraction reagents, Thermo Scientific, with reference to Tsai et al. 2010), although acquiring a completely pure fraction will be challenging.

6.4 Nictaba is transported into the nucleus

Nictaba is a nucleocytoplasmic protein. The distribution of the lectin in the cytoplasm and the nucleus was confirmed in Chapter 3 by stable and transient transformation techniques in both tobacco plants and suspension cells. The mechanisms responsible for transport of the lectin into the nucleus were scrutinized (Figure 6.1-3). In 2006, Lannoo et al. showed that the monopartite NLS present in the Nictaba sequence was sufficient and necessary for nuclear import (Lannoo et al. 2006b). These results could not be confirmed, since the Nictaba protein mutated in its NLS was still transferred into the nucleus after Agrobacterium-mediated transformation. Complete diffusion is unlikely, since the localization pattern of free EGFP is clearly different from the one of EGFP-Nictaba. Possibly, multiple pathways are responsible for the nuclear import. For example, a combination of active transport and passive diffusion as seen for galectin-3 (Nakahara and Raz 2007) or Nictaba uses a novel pathway like β-catenin to enter the nucleus (Suh and Gumbiner 2003). In future research, it should be clarified how nuclear import is mediated. The functionality of the NLS can be checked by
fusing EGFP-Nictaba with a large non-nuclear protein e.g. GUS and monitoring its nuclear import. The discrepancy between results can also be evaluated by micro-injection of the wheat germ agglutinin (WGA) into the cytoplasm of the cell. This lectin binds to proteins of the nuclear pore complex and selectively blocks carrier-mediated transport without interfering with passive diffusion or alternative pathways (Davis 1995, Liashkovich et al. 2012). WGA recognizes the O-GlcNAc residues present on numerous nuclear pore proteins (NUPs) located in the periphery of the nuclear pore complex. O-GlcNAc is presumably involved in nuclear transport but this process is poorly understood. However, the O-GlcNAcylation of NUPs is believed not to have an active role in nuclear translocation. O-GlcNAc residues on the cargos themselves rather than the NLS were suggested to be a signal for nuclear residence (Guinez et al. 2005).

Nictaba has a high affinity towards O-GlcNAc moieties. The putative involvement of this sugar-binding activity in nuclear transport was assessed by evaluating the localization of a Nictaba protein lacking carbohydrate binding activity (Chapter 3). Neither the NLS sequence (see above) nor the lectin activity seemed to be involved in the nuclear transport of Nictaba. However, it remains unclear how the nuclear transport happens. Two different mechanisms can be proposed: nuclear import by an alternative NLS-independent transport pathway or by passive diffusion through the NPC. The previously suggested WGA experiment should give some ideas and can possibly confirm or exclude passive diffusion.

Whether Nictaba is dynamically shuttling between the nucleus and the cytoplasm is not clear. Leptomycin treatment executed in Chapter 3 did not influence the localization pattern of Nictaba, indicating two possibilities: (a) Nictaba does not dynamically shuttle between the nucleus and the cytoplasm or (b) Nictaba does not need a NES sequence for nuclear export. The latter possibility seems to be the most likely one. Proteins lacking a NES sequence can also be exported from the nucleus by specific exportins, although this export is connected with substrate phosphorylation (Sorokin et al. 2007). Furthermore, nuclear export can also happen through passive diffusion.

6.5 Occurrence of glycosylated proteins in the nucleus

Glycan array analysis showed that Nictaba specifically interacts with GlcNAc-(oligomers) and with the Man$_3$GlcNAc$_2$-core of high mannose and complex N-glycans (Lannoo et al. 2006b). By now evidence is growing for the occurrence of glycosylated proteins in the nucleus and the cytoplasm and especially O-GlcNAc is seen as a common PTM of proteins occurring in the nucleus and/or cytoplasm. However, the majority of the research was carried out in mammals and much less is known about glycosylation in these cellular compartments in plants. In Chapter 4, we confirmed the presence of O-GlcNAc on nuclear proteins in plants and identify this modification on histone proteins. Recently, O-
GlcNAcylation of histone proteins was also detected in animal systems, but to our knowledge this is the first time histone glycosylation was found in plants. In animal cells, O-GlcNAc was shown to serve as a stress sensor which triggers different processes such as changing gene expression. Furthermore, it was shown that O-GlcNAcylation of histones is cell cycle dependent in animal cells, which was confirmed in our analyses for plant cells. These data suggest that O-GlcNAc might serve as a checkpoint for cell cycle progression, similar to phosphorylation of core histones. Since the O-GlcNAcylation of core histones is a very recent discovery, it remains unclear what its precise function is and more research will be needed to elucidate this phenomenon.

6.6 Nictaba interacts with histones in the nucleus

Once Nictaba has entered the nucleus, the lectin is distributed throughout the entire nucleus (Figure 6.1-4) except the nucleolus. Nictaba was shown to be very mobile within the nucleus similar to nuclear proteins transiently interacting with chromatin (Chapter 3). Furthermore, the lectin is found especially at the periphery of the nucleus. Whether Nictaba is associated within the nuclear envelope could not be clarified. However, Nictaba seems to be highly enriched in that region. Electron microscopy using immunogold labeling of EGFP or Nictaba could give a decisive answer about the exact localization. According to the conventional chromatin architecture especially gene-poor domains localize at the nuclear periphery, whereas the interior regions of the nucleus are preferentially occupied by gene-rich regions. However, exceptions to this model of nuclear architecture exist, such as in *Saccharomyces cerevisiae*, where the highly transcribed GAL1 gene associates with the nuclear pore complexes (Bartova et al. 2008).

It was suggested that Nictaba was transported within the nucleus by diffusion to be able to cover the entire nucleus (Chapter 3). Inside the nucleus, Nictaba was shown to interact with the core histone proteins using a proteomics approach (Schouppe et al. 2011). These findings were confirmed *in vitro* using plant histone proteins, whereas *in vivo* studies using BiFC and FRET could not confirm nor reject this interaction (Chapter 5). Additional experiments such as FLIM and SPR could help to confirm the association between Nictaba and the core histones.

Through the putative interaction with histones, Nictaba can associate with euchromatin or heterochromatin. Since Nictaba is induced upon stress, it seems rather unlikely that the lectin interacts with heterochromatin which is more correlated with differential gene expression during development or cellular differentiation (Bannister et al. 2011). Nictaba is presumably associated with euchromatin and might be involved in activating or silencing transcription. It is tempting to speculate that the recognition of the O-GlcNAc modification on histone proteins by Nictaba is similar to the chromodomains and bromodomains recognizing histone methylation and acetylation, respectively,
and tethers different protein complexes onto chromatin. These complexes can have various functions, such as specific enzymatic activities to remodel chromatin or to prevent other complexes to bind, which eventually will influence transcription.

To further elucidate the interaction between Nictaba and histones and the effects resulting therefrom, more experiments are needed. First of all, a chromatin immunoprecipitation experiment would be very helpful to identify the chromatin regions which are associated with Nictaba. Characterization of the histone modifications can be useful to check whether this chromatin region belongs to euchromatin or heterochromatin. Also the identification of the genomic DNA sequences which are bound by Nictaba would be of great interest to further elucidate the function of Nictaba. If Nictaba is involved in gene regulation, a micro-array experiment could give an idea of which genes are up- or down-regulated in the absence or presence of the lectin.

6.7 Conclusion

In this work, research was performed to meet with some predefined objectives in an attempt to elucidate the physiological role of Nictaba. The tissue-specific expression of the lectin in transgenic Arabidopsis and tobacco plants was analyzed and compared. Also several jasmonate responsive elements were identified in the promoter sequence of Nictaba (objective 1). Subcellular localization of Nictaba in plants and suspension cells was investigated. Surprisingly, we found that nuclear import was independent of the NLS and the carbohydrate activity of Nictaba. No complete answer was found for the mobility of the lectin in the nucleus (objective 2). Both the presence of the O-GlcNAc modification on tobacco histones and the cell cycle dependency of this O-GlcNAc modification have been shown (objective 3). Finally, the interaction between Nictaba and histones was confirmed in vitro but could not irrefutably be confirmed in vivo (objective 4).
Appendix A
<table>
<thead>
<tr>
<th>Primer</th>
<th>5’ – 3’ sequence</th>
<th>Amplification product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evd 1</td>
<td>AAAAACAGGGCTTCACCATGCAAGGCCAGTGATAGCCGC</td>
<td>Forward primer to amplify Nictaba (AF389848) and first part of attB1</td>
</tr>
<tr>
<td>Evd 2</td>
<td>GGGGACAAGTTGTAACAAAAAGCAGGCT</td>
<td>Primer to complete the attB1-site</td>
</tr>
<tr>
<td>Evd 3</td>
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<td>Reverse primer to amplify Nictaba (AF389848) and first part of attB2</td>
</tr>
<tr>
<td>Evd 4</td>
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</tr>
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<td>Reverse primer to amplify 3’ UTR of Nictaba</td>
</tr>
<tr>
<td>Evd 65</td>
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</tr>
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</tr>
<tr>
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<td>Evd</td>
<td>Forward/Reverse Primer</td>
<td>Description</td>
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</tr>
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</tr>
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</tr>
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<td>GUS-RV</td>
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<td>Reverse primer to amplify GUS gene</td>
</tr>
</tbody>
</table>
Appendix B
Figure B Co-localization of EGFP-Nictaba with the different histone proteins fused with RFP. Panel 1 shows the localization of EGFP-Nictaba, panel 2 shows the localization of the different histones and panel 3 shows the overlay image of panels 1 and 2. A, RFP-H2A; B, H2A-RFP; C, RFP-H2B; D, RFP-H4; E, H4-RFP. The scale bar is 25 µm.
Appendix C
Legend on page 166.
Figure C Confocal microscopy images of transiently transformed *N. benthamiana* leaves expressing Nictaba coupled with CFP\textsuperscript{C} and histone proteins coupled with VENUS\textsuperscript{N}. Interaction of these proteins gives an emission of green fluorescent light upon excitation (1). The red dots show the autofluorescence of the chloroplasts (2). The transmission image shows the surface of the leaves with the typical epidermis cells (3). In the right panel, all the images were merged and the complete picture is shown (4). A, SCYCE-Nictaba + VYNE-H2A; B, SCYCE-Nictaba + H2A-VYNE; C, SCYCE-Nictaba + VYNE-H2B; D, SCYCE-Nictaba + H2B-VYNE; E, SYCE-Nictaba + H4-VYNE; F, Nictaba-SCYCE + VYNE-H2A; G, Nictaba-SCYCE + H2A-VYNE; H, Nictaba-SCYCE + VYNE-H2B; I, Nictaba-SCYCE + H2B-VYNE; J, Nictaba-SCYCE + VYNE-H4; K, Nictaba-SCYCE + H4-VYNE, Scale bar is 25 \( \mu \)m.
Summary/Samenvatting
Plant lectins are defined as “all plant proteins that possess at least one non-catalytic domain that binds reversibly to a specific mono- or oligosaccharide”. For a long time, plant lectin research has concentrated on lectins found in seeds and vegetative storage tissues. These lectins are constitutively expressed in the plant and are targeted via the secretory pathway into the vacuolar or extracellular compartment. It was shown that many of these lectins combine a defense-related role against predators with a function as a storage protein. In the last decade, a new class of lectins was identified, which are only expressed after exposure of the plant to stress and changing environmental conditions. These stress-related lectins are expressed in low concentrations and reside in the nucleocytoplasmic compartment of the plant cell. Consequently, it was proposed that some of these lectins are involved in endogenous signaling events induced in the plant upon stress.

The research performed in this work focuses on elucidating the physiological role of one particular nucleocytoplasmic lectin, the *N. tabacum* agglutinin or Nictaba. Nictaba is expressed in tobacco leaves treated with jasmonates or subjected to insect herbivory. Therefore, it is believed that Nictaba acts as a signaling protein involved in the stress physiology of the plant. In the first chapter an overview is given of current literature concerning the tobacco lectin.

In chapter 2, experiments were executed using *A. thaliana* and *N. tabacum* plants stably expressing a promoter-GUS fusion construct to gain more knowledge with respect to the tissue-specific expression of the jasmonate-inducible lectin during plant development. In Arabidopsis, a strong GUS staining pattern was detected in very young tissues (the apical and root meristems, the cotyledons and the first true leaves), but the promoter activity decreased when plants were getting older. Both histochemical and fluorometric techniques were used to follow Nictaba promoter activity during the development of the tobacco plants. GUS staining was predominantly detected in the cotyledons, the leaves and the roots during the youngest plant stages. As the plants grow older GUS staining was mostly present in the older leaves. A detailed comparative analysis was made of the GUS staining results obtained in transgenic Arabidopsis and tobacco lines. Furthermore, Nictaba expression was also detected at low concentrations in tobacco roots and expression levels increased after cold treatment.

Molecular cloning of the lectin cDNA and sequence analysis revealed the absence of a signal peptide and the presence of a typical monopartite nuclear localization signal (NLS) KKKK, suggesting that the protein could locate to the nucleus. Immunolocalization studies revealed the nucleocytoplasmic localization of Nictaba in jasmonate treated tobacco leaves. Previous localization studies using EGFP-tagged fusion proteins, which were transiently expressed in tobacco BY-2 suspension cells by particle bombardment, indicated that the NLS is required and sufficient for transporting Nictaba from the
In chapter 3, these localization studies were extended to stably transformed suspension cells as well as transiently and stably transformed N. benthamiana leaf cells. Three different EGFP constructs were included in the analysis, being the native Nictaba sequence, an NLS mutant (K^{102}TAK^{105}) and a mutant affected in the carbohydrate binding site (W^{15}L^{15}). No differences were observed in the localization patterns for Nictaba between suspension cells and leaf cells being transiently or stably transformed with the different EGFP constructs. Unexpectedly, the localization pattern for Nictaba and the two mutant proteins was similar, indicating that neither the NLS nor the carbohydrate binding activity of the protein are necessary for nuclear import.

In previous research, a nuclear proteomics approach was followed to search for binding partners of Nictaba. Using a pull-down assay and lectin affinity chromatography with N. tabacum cv Xanthi nuclei, histones were identified as primary interaction partners for Nictaba. This interaction was confirmed in vitro by affinity chromatography of purified calf thymus histone proteins on a Nictaba column and occurred by binding of the lectin to the O-GlcNAc modification present on the calf histones. The O-GlcNAc modification of histones in mammalian cells was discovered very recently and was found to be cell cycle dependent. Hereupon, the question was raised whether this modification also occurs in plants and is cell cycle related. Therefore, in chapter 4, tobacco BY-2 and Xanthi suspension cells were synchronized using aphidicolin drug treatment. Transcript expression for the O-GlcNAc transferase gene and the Nictaba gene were monitored through cell cycle progression. Concomitantly, O-GlcNAcylation of histone proteins was studied. We showed for the first time that also plants histones are modified by O-GlcNAc and that this modification is cell cycle dependent. Nictaba and O-GlcNAcylated histones presumably interact during S- and/or G2-phase, since at this time they are both present within the nucleus. It is suggested that this interaction allows Nictaba to act as a modulator of gene transcription.

In chapter 5, the interaction between Nictaba and tobacco histones is scrutinized both in vitro and in vivo. To this end, a protocol was developed to purify histones from tobacco BY-2 suspension cells. Using lectin chromatography and far Western blot analysis, the interaction between tobacco histones and Nictaba was confirmed. Furthermore, co-localization studies showed that both binding partners reside in the same cellular compartment, the nucleus. To assess the interaction in vivo, bimolecular fluorescence complementation and fluorescence resonance energy transfer experiments were performed. The results of both microscopic techniques could not irrefutably confirm the interaction between Nictaba and histones.
Plantlectinen worden gedefinieerd als “alle planteiwitten die minstens één niet-katalytisch domein bevatten dat reversibel kan binden met een specifiek mono- of oligosaccharide”. Het onderzoek naar plantlectinen heeft zich lange tijd geconcentreerd op lectines gevonden in zaden en vegetatieve opslagweefsels. Deze lectinen komen constitutief tot expressie in de plant en worden via de secretorische pathway getarget naar de vacuole of het extracellulaire compartiment. Er werd aangetoond dat veel van deze lectines een verdedigingsgerelateerde functie tegen predatoren combineren met een functie als opslageiwit. In het latste decennium werd een nieuwe klasse van lectinen geïdentificeerd, die enkel tot expressie komen als de plant wordt blootgesteld aan stress of wisselende omgevingscondities. Deze stressgerelateerde lectines komen in lage concentraties tot expressie en zijn aanwezig in het nucleocytoplasmatische compartiment van de plantencel. Bijgevolg werd voorgesteld dat sommige van deze lectines betrokken zijn in endogene signaalreacties die ontstaan in de plant als een respons op een stresssituatie.

Het onderzoek verricht in dit werk focust op het verduidelijken van de fysiologische rol van één specifiek nucleocytoplasmatisch lectine, het *N. tabacum* agglutinine of Nictaba. Nictaba komt tot expressie in tabaksbladeren behandeld met jasmijnzuur of onderworpen aan insectenvraat. Daarom wordt aangenomen dat Nictaba functioneert als een signaaleiwit betrokken in de stressfysiologie van de plant. In het eerste hoofdstuk wordt een overzicht gegeven van de literatuur met betrekking tot het tabakslectine.

In hoofdstuk 2 werden experimenten uitgevoerd gebruik makende van *A. thaliana* en *N. tabacum* planten die een promoter-GUS fusieconstruct tot expressie brengen, om meer inzicht te krijgen in de weefselspecifieke expressie van het jasmijnzuur geïnduceerde lectine tijdens de ontwikkeling van de plant. In Arabidopsis werd een sterke GUS-kleuring gedetecteerd in de heel jonge weefsels (het apicaal- en wortelmeristeem, de cotyledonen en de eerste echte bladeren), maar de promoteractiviteit daalde naarmate de planten ouder werden. Zowel histochemische als fluorometrische technieken werden gebruikt om de activiteit van de Nictabapromoter te volgen tijdens de ontwikkeling van de tabaksplanten. Tijdens de jongste plantstadia was GUS-kleuring voornamelijk aanwezig in de cotyledonen, de bladeren en de wortels. Wanneer de planten ouder werden was er vooral GUS-kleuring in de oudere bladeren. Er werd een gedetailleerde vergelijkende analyse gemaakt van de GUS-kleuringsresultaten verkregen in transgene Arabidopsis en tabakslijnen. Bovendien werd Nictaba expressie ook in lage concentraties gedetecteerd in de wortels van tabak en stegen de expressieniveaus na een koudebehandeling.

Moleculair klonen van het lectine cDNA en sequentieanalyse toonden de afwezigheid van een signaalpeptide en de aanwezigheid van een typisch ééndeling nucleair lokalisatiesignaal (NLS) KKKK
aan, wat erop wijst dat het eiwit aanwezig kan zijn in de nucleus. Immunolokalisatiestudies toonden de nucleocyttoplasmatische lokalisatie van Nictaba aan in de bladcellen van met jasmijnzuur behandelde tabakspellen. Vroeger uitgevoerde lokalisatiestudies met EGFP-gelabelde fusie-eiwitten, die transient tot expressie werden gebracht in BY-2 suspensiecellen door middel van deeltjesbombardement, gaven aan dat de NLS vereist en voldoende is om Nictaba vanuit het cytoplasma naar de kern te transporteren. In hoofdstuk 3, werden de lokalisatiestudies uitgebreid met stabiel getransformeerde suspensiecellen en zowel transient als stabiel getransformeerde N. benthamiana bladcellen. Drie verschillende EGFP constructen werden opgenomen in deze analyse, namelijk de natieve Nictaba sequentie, een NLS mutant (K102KTAK105) en een mutant met een gewijzigde suikerbindingsplaats (W15→L15). Er werden geen verschillen waargenomen tussen suspensiecellen en bladcellen na stabiele of transiente transformatie met de drie EGFP-constructen. Het lokalisatiepatroon van Nictaba was gelijkaardig aan dat van de twee mutanten, wat aangeeft dat noch de NLS noch de suikerbindingsactiviteit van het eiwit noodzakelijk zijn voor import in de kern.


In hoofdstuk 5 werd de interactie tussen Nictaba en de tabakshistonen zowel in vitro als in vivo in detail onderzocht. Hiervoor werd een protocol ontwikkeld om histonen op te zuiveren uit een BY-2 suspensiecultuur. De interactie tussen tabakshistonen en Nictaba werd bevestigd met behulp van lectine affiniteitschromatografie en far Western blot analyse. Bovendien toonden co-lokalisatiestudies aan dat beide bindingspartners aanwezig zijn in hetzelfde cellulare compartiment,
Summary/Samenvatting
	namelijk de kern. Om de interactie in vivo te bepalen werden bimoleculaire fluorescentie complementatie en fluorescentie resonantie energietransfer experimenten uitgevoerd. De resultaten van beide microscopische technieken laten echter niet toe om op een onweerlegbare manier de interactie tussen Nictaba en histonen te bevestigen.
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Curriculum vitae

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Meetings without presentation

- Joint meeting COST action/Groupe de Contact FNRS Proteomique “Systems biology and Omic approaches” May 7th, 2010 at Namur
- Advanced Light Microscopy Symposium September 23-24, 2012 at Ghent
- Belgian Society of Biochemistry and Molecular Biology " Proteomics and interactomics in signaling networks " May 25th, 2012 at the University of Ghent