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Isolation and Characterization of Sequences Homologous to the Tobacco Clone *Axi 1* (auxin independent) from a *Vicia sativa* Nodule cDNA Library

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Abstract: In this research, partial nucleotide sequences of the *axi 1* gene, which is related to auxin perception and transduction, isolated from *Vicia sativa* using cDNA library screening were investigated. Four *V. sativa* cDNA clones representing homologous of the tobacco *axi 1* (auxin independent) cDNA clone were isolated and characterized. With sequence analysis two different clones were observed, called 10/3a and 16/3b. Sequences about 500 bp from the 5' end and 450 bp from the 3' end were determined from the 10/3a clone. The sequences from the 5' end of cDNA clone 10/3a shared about 58 % similarity at the nucleotide level and 61% identity at the polypeptide level with the sequences of the *Nicotiana tabacum axi 1* cDNA clone. At the 3' end of 10/3a, 63 % similarity at the nucleotide level was observed with the clone. In 16/3b about 500 bp was sequenced from the 5' end. There was 64 % nucleotide sequence similarity and 61 % polypeptide sequence identity. Northern blot analysis showed that the size of the mRNA was about 2 kb, like that of the *axi 1* isolated from tobacco. It was shown that homolog sequences of *axi 1* were expressed in flowers, leaves, stems, roots and nodules of the *V. sativa* plants.

Key Words: *axi 1*, auxin, gene isolation, *Vicia sativa*

Fiğ (*Vicia sativa*) Bitkisinin Nodül cDNA Kütüphanesinden, Tütün *Axi 1* (Oksinden Bağımsız) Klonunun Homolog Dizinlerinin İzole Edilmesi ve Karakterizasyonu

Özet: Bu araştırmada, fiğ (*Vicia sativa*) bitkisinin cDNA kütüpanesinden, oksin alımı ve kullanımından sorumlu olan *axi 1* geninin kısmi nükleotid dizinleri araştırılmıştır. Tütünden izole edilmiş olan *axi 1* (oksinden bağımsız) geni prob olarak kullanılarak bu gene benzerlik gösteren dört *V. sativa* cDNA klonu izole edilmiş ve kısmi gen dizinleri karakterize edilmiştir. Dizin analiz yöntemi ile 10/3a ve 16/3b olarak adlandırılan iki farklı cDNA klonu saptanmıştır. 10/3a cDNA klonunun 3 ' ucundan 450 baz çifti, 5 ' ucundan ise yaklaşık 500 baz çifti belirlenmiştir. 10/3 a cDNA klonunun 5 ' ucu, *Nicotiana tabacum axi 1* cDNA klonu ile nükleotid düzeyinde % 58, polipeptid düzeyinde ise % 61; 3 ' ucu ise nükleotid düzeyinde % 63 oranında benzerlik göstermiştir. 16/ 3b cDNA klonunun 5 ' ucundan ise 500 baz çifti elde edilmiş ve *Nicotiana tabacum axi 1* cDNA klonu ile nükleotid düzeyinde % 64, polipeptid düzeyinde ise % 61 oranında benzerlik saptanmıştır. Northern blot analizi: *V. sativa axi 1* mRNA'sının büyüklüğünün tütünden izole edilmiş *axi 1* gibi 2 kilobaz çiftinde olduğunu ve bu genin bitkinin çiçek, yaprak, gövde, kök ve nodül dokularında sentezlendiğini ortaya çıkarmıştır.

Anahtar Sözcükler: *axi 1*, gen izolasyonu, oksin, *Vicia sativa*

Introduction

In the plant kingdom, a great variety of pathogenic, saprophytic and symbiotic interactions between plants and microorganisms occur, and several of these interactions have been the subject of intensive research. One of the best studied interactions is the symbiosis of *Rhizobium* bacteria and legume plants. The symbiosis results in the formation of root nodules, in which bacteria are able to fix atmospheric dinitrogen into ammonia, a process called symbiotic nitrogen fixation (Franssen et al., 1992).

An initial step in legume nodulation is root hair deformation in the receptive part of the root, and the induction of cell division in the fully differentiated root

cortex of the plant. These cortical cells are mitotically activated by specific lipo-oligosaccharides called Nod factors, which are secreted by *Rhizobium* (Denaire et al., 1992). It has been shown that nodule specific plant genes (nodulin genes) are induced in the dividing cortical cells, namely ENOD 12 and ENOD 40. The expression of both early nodulin genes is induced in dividing root cortical cells (Scheres et al., 1990, Yang et al., 1994), and in addition ENOD 40 is induced in the region of the pericycle of the root opposite the dividing cortical cells (Yang et al., 1994). Purified Nod factors have the ability to induce cell divisions in the cortex of leguminous plants, and in vetch ENOD 40 as well as ENOD 12 are induced in these nodule primordia (Vijn et al., 1993).

ENOD 40 is one of the plant genes active during nodule formation and function. ENOD 40 clones have been isolated from various leguminous plants, e.g. soybean GmENOD40 (Kouchi and Hata, 1993), GmENOD40-2 (Yang et al., 1993) pea PsENOD40 (Matvienko et al., 1994) and vetch VsENOD40 (Vijn et al., 1995). Recently the first ENOD 40 clones from a non-legume were isolated, the tobacco clones NtENOD40-1 and –2 (Matvienko et al., 1996, Van de Sande et al., 1996). Transient and stable expression of ENOD40 in tobacco protoplasts induced tolerance of supra-optimal auxin concentrations. Whereas the division frequency of wildtype protoplasts decreased at supraoptimal NAA concentrations, ENOD40 expression enabled the protoplasts to divide with unreduced frequency.

Lipochitin oligosaccharides, rhizobial signalling molecules capable of triggering several plant responses involved in nodulation (Denaire et al., Vijn et al.), have been postulated to influence the plant growth regulator balance in legume roots (Myolona et al.). Synthetic lipochitin oligosaccharides (LCOs) have also been tested for their effect on tobacco protoplast division. They were able to induce tobacco protoplast division in the absence of auxin or cytokinin (Van de Sande, 1997).

Studies on auxin transport inhibitors as well as experiments on the role of ENOD 40 genes in nodulation showed that phytohormones and particularly auxin might play a crucial role in the early stages of nodule development. Auxin transport inhibitors, probably leading to a modified auxin distribution in the plant, induce the formation of nodule-like structures, in which early nodulin genes are expressed (Asad et al., 1994, Hirsch et al., 1989, Scheres et al., 1992, Wu et al., 1996). According to Libbenga (1973), when stele free pea root explants were cultured on medium containing auxin and cytokinin, cell divisions were induced in the inner cortical cell layers, preferentially opposite the former location of the protoxyleme poles.

According to Walden (1994), the *axi 1* (auxin independent) gene that has been tagged in *axi 1*59 and whose deregulated expression results in auxin independent growth *in vitro* was characterized. It was shown that *axi 1* conferred on protoplasts the ability to grow in culture not only in the absence of auxin but also at high levels of auxin concentration where cell division is not observed in wild type protoplasts. So far, tobacco and Arabidopsis plants are used as a model system to understand the biological effect of the *axi 1* gene in plant metabolism. *Axi 1* is the only characterized auxin independent gene in tobacco plants (Hayashi et al., 1992).

The purpose of this research was to determine the nitrogen fixation mechanism to determine related genes which transfer this character to the plants that are not able to fix nitrogen but are commercially important. In this research, partial nucleotide sequences of the *axi 1* gene, which is related to auxin perception and transduction isolated from *Vicia sativa* using cDNA library screening were investigated.

Materials and Methods

Plant Materials and Bacterial Strains

In this study, *V. sativa* (vetch) and *Rhizobium leguminosarum* bv. PRE strain were used as plant and bacterial materials, respectively.

During the experiments, Escherichia coli strains, XL - Blue and DH5 α were used for transformation and propagation of the constructs. PBluescript II +/- phagemid plasmid was also used for cloning.

Methods

DNA Sequencing

The homologous sequences to the *axi 1* gene isolated from tobacco were isolated from the *V. sativa* nodule cDNA library (Vijn et al., 1995) using library screening techniques (Sambrook et al., 1989). These isolated homologous sequences located in the lambda ZAP II vector were seperated from this vector by *in vivo* excision

(Sambrook et al., 1989) and cloned into pBluescript II KS +/- phagemid plasmid. After phage amplification and *in vivo* excision of the pBluescript containing the insert fragment, plasmid DNA was isolated by the alkaline lysis miniprep method and transformed into DH5 α cells. The *in vivo* excised and transformed cells were used again for DNA isolation. Nucleotide sequences were determined by dideoxy chain termination with a T7 Sequencing TM kit (Sambrook et al., 1989). DNA and RNA structures were examined in detail by southern and northern blot analysis.

Southern Blots

The isolated plasmids from each positive phage were digested with the restriction enzymes (*EcoRI* and *Not I*) with which the library was made and cross hybridized with a 900 bp *PstI* and *HindIII* fragment of the *axi 1* cDNA clone from tobacco (Walden et al., 1994). This revealed the cloned inserts from the pBluescript plasmid. The fragments were visible after DNA electrophoresis in an agarose gel (1%) and blotted onto geneScreen in 0.025 M NaH_2PO_4 pH 7.0. The isolated fragment from positive clone *axi 1* (16/3b) was labelled by random priming using (α 32P) dATP (3000 Ci/mmol) as radioactive label.

Northern Blots

Total RNA was isolated from 8-day-old leaves, stems, roots and flowers collected from uninoculated *V. sativa* plants and 12-day-old nodules inoculated with *R. leguminosarum* bv vicia PRE strain and denaturated in DMSO/glyoxal, separated on 1% agarose gels and blotted on to geneScreen in 0.025 M NaH_2PO_4, pH 7.0. The blots were hybridized in 50% formamide, 1M NaCl, 0.5% SDS, 10mM Tris HCl pH 7.0, 10x Denhards solution at 42ºC. The isolated fragment from the positive clone *axi 1* (16/3b) was labelled by random priming using (α 32P) dATP (3000 Ci/mmol) as radioactive label.

Results and Discussion

Southern blots revealed the cloned inserts from the pBluescript plasmid. In Figure 1, DNA electrophoresis of the clones 7a, 10/3a, 10/5d and 16/3b after digestion with *EcoRI* and *NotI* (a) and southern blot results from the digested clones (b) are shown. Two fragments of about 1.2 kb and 200 bp for clone 7a, about 800 bp and 350 bp for clone 10/3a, about 1.2 kb for 10/5d and about 1.2 kb for clone 16/3b can also be seen. The fact

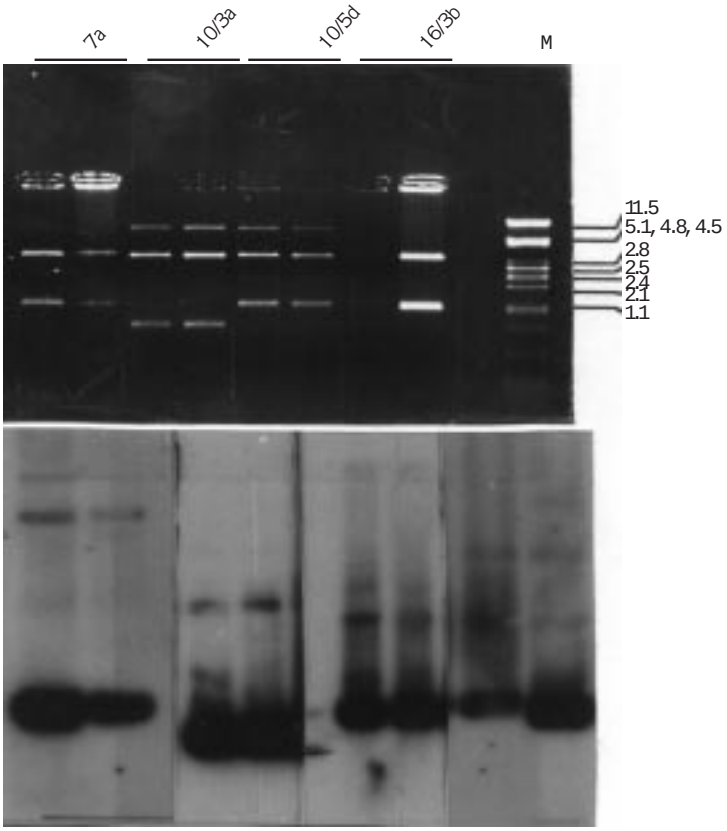

Figure 1. DNA electrophoresis of clones 7a, 10/3a, 10/5d and 16/3b after digestion with *EcoRI* and *NotI* (a) and southern blot results from the digested clones (b).

that more fragments are seen indicates that there are internal *EcoRI* and/or *NotI* sites in the cDNA clones. The band at about 9 kb probably represents a leftover concatamer. As a probe, the 900 bp *PstI* and *HindIII* fragment of the *axi 1* cDNA clone from tobacco was used. This fragment showed cross hybridization with the 1.2 kb band from clones 7a, 10/5d and 16/3b and the 800 bp fragment from clone 10/3a.

Northern blot analysis revealed that the overall expression level was rather low, but in roots and nodules, the expression was the strongest. Slightly weaker expression was detected in the flowers, and the expression in stems and leaves was also slightly lower.

It has been reported that *axi 1* was expected only in the roots of wild type plants and a certain level of external auxin was required for cell division to occur in protoplasts isolated from wild type plants (Hayashi et al., 1992). Analysis showed no signal in RNA from leaf tissue and callus although from time to time, a weak signal was also observed in RNA isolated from shoot apices. In the tagged plant line, *axi 1*59, *axi 1* RNA can be detected in all tissue tested, indicating that *axi 1* expression is activated apparently in a tissue-independent manner (Walden et al., 1994). In protoplasts isolated from both wild type and *axi 1*59 plants cultured in the presence of auxin, the *axi 1* transcript became detectable 2-3 days following isolation. Transgenic *axi 1*59 plants expressed *axi 1* constitutively and protoplasts isolated from these plants did not require external auxin for cell division to occur.

Lipochitin oligosaccharides (LCO), rhizobial signalling molecules capable of triggering several plant responses involved in nodulation (Denaire et al., Vijn et al.), have been postulated to influence the plant growth regulator balance in legume roots (Myolona et al.). Most probably, LCOs activate cell division by a different signal transduction pathway than auxins, since agonists for NAA and LCOs are only able to block divisions induced by their structural analogue. At a certain point both pathways seem to converge, because both LCOs and NAA induce *axi 1* expression, which precedes cell division. The common part of the signal transduction pathway is influenced by cytokinin since the addition of cytokinin was required to obtain maximal *axi 1* expression in the presence of either auxin or LCOs. Thus, in protoplasts, *axi 1* expression is part of the signal transduction pathways started by either auxin or LCOs in cooperation with cytokinin (Röhrig et al., Van de Sande, 1997).

Our findings in *V. sativa* that *axi 1* is expressed in all organs investigated, although at varying, low levels, suggest a threshold level of endogeneous levels of auxin of induce the *axi 1* gene. There appeared to be an absolute requirement for the presence of auxin in the medium to induce these cell divisions, but cell divisions were also greatly stimulated by the addition of cytokinin. The role of cytokinin in nodule initiation was shown (Cooper and Long, 1994). Cytokinin induces not only cell division in the inner cortex of legume roots, but also expression of ENOD 40 in the part of the pericycle opposite the dividing cells. Studies on *axi 1* and LCOs show that auxin and cytokinin signal transduction are linked. Furthermore, cytokinin and LCOs are signal molecules implicated in nodule formation, where both induce cell division and ENOD 40 expression.

Northern blot analysis showed that size of the mRNA was about 2 kb, like that of the *axi 1* isolated from tobacco. A 2.1 kb cDNA was synthesized from polyadenylate (poly A) RNA isolated from leaf tissue of *axi 1*59. The deduced amino acid sequence from the cDNA reveals a highly basic protein and confers the ability of protoplasts to divide *in vitro* in the absence of auxin (Hayashi et al., 1992).

Sequence analysis revealed that so far two different clones homologous to the tobacco clone *axi 1* cDNA clone were identified in *V. sativa*, namely 10/3a and 16/3b. From clone 16/3b, the 5' end was sequenced (Figure 2). There was 64 % nucleotide sequence similarity and 61% polypeptide sequence identity with that of the *Nicotiana tabacum axi 1* cDNA clone obtained by Walden et al. (Walden et al., 1994). From clone 10/3a, parts from the 5'end and the 3' end were sequenced (Figures 3 and 4). The sequences from the 5' end of the cDNA clone 10/3a shared about 58 % similarity at the nucleotide level and 61 % identity at the polypeptide level with the sequences of the *Nicotiana tabacum axi 1* cDNA clone. At the 3' end of 10/3a, 63 % similarity at the nucleotide level was observed with the *axi 1* clone. The 3' end from clone 10/3a was homologous to the 5' end from 16/3b. The nucleotide sequence from the 5' ends of 16/3b and 10/5d were identical, and therefore 10/5d was not used in further analysis. Sequencing of clone 7a was not possible.

The sequences from two clones showed significant homology, but also some differences. To get complete sequences from the isolated clones and to be able to sequence the entire clone from two directions with

different restriction enzymes and subcloning into pBluescript KS +/- are currently being carried out. However the sequence data have not been obtained to date.

On the other hand, to understand the involvement of *axi 1* in root nodule organogenesis, the changes of internal auxin levels in relation to *axi 1* expression and also releated proteins, as well as the effects of exogeneous auxins should be determined. It is worth looking at the effect of nod factors on the expression of *axi 1* gene in legume plants, as well as the relationship between the *axi 1* expression and ENOD gene expression. Perhaps one of the most important aspects of the *axi 1* expression will be determined by *in situ* hybridisation in legume plants.

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1  TCGAATTCCGTTGCTGTCGCCGTTGNTGTCGCCGTTGCTGTCGAAAGAAA
51 CCATTACTACCAAAGAAGAATTAGAACCATTTTCGTCGTTTTCTTCTCGT
101ATGGCTGCACTTGATTTTATTGTTTGTATGAAAGCGACGTGTTTGTTCAC
151CAATAACAATGGTAACATGGGTAAATATTAGCTGGGCGAAGGAGATACT
201TTGGGCACAAACCAACCATTCGTCCAAATGCTAAAAAACTCTACAGATTG
251TTCATGAACAGAAGTAATTTGACTTGGGATGTTTGTGCTTCCAGTATACG
301TACCTTCCAGAAAGGATTCATGGGTGAGCCAAAAGAGCTTAGACCTGGTA
351GAGGTGGGTTTCATGAAAATCCATCTACTTGCATATGTGAAGATTCTGTG
401GAGAAAGCGGCCAAAAATTCTGGACCTAGAAAA
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Figure 2. Nucleotide sequence of the 5' end from *EcoRI/NotI* subcloned cDNA fragment from the 16/3b clone.

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1  CGAAGTCGACGGTATCGATAAGCTTGATATCGAATTCCGTTGCTGTCGGC
51 CAATGACCTCGATGATGAGCTACAAAAACTCCGTTGCCGTGTTAACTATC
101ATGCTTTGAGATTTACAAAACCTATACGAAAACTTGGTCAAAAAATTGTC
151ATGAGAATGCAAAAGATGGCAAGCCGTTACATAGCAGTTCATNTAAGGTT
201TGAGCCAGATATCGTGGCATTTTCAGGTTGTTATTTTGGTGGGGGTGAGA
251AAGAAAAACAAGAGCTTGGTGAAATCAGAAAAAGATGGACAACACTGCT
301GATTTGAGCCCTGATGAAGAGCGAAAGCGTGGGAAATGTCCTCTTACTCC
351TCACGAAGTGGGTTTGATGCTGCGAGCACTTGGTTTTACAAATGACACAT
401ACTTATTTGTTGCGTCGGGAGAAATATATGGCGGGGGATGGAAACCATGC
451AGCCTCTTAAAGGAACTTTTCCCCAACATCTATACAAAGGGAGGATGCTC
501GCTGAAGCAAAGCTGAAACCTTCCCCTTTCATTTTCTT
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Figure 3. Nucleotide sequence of the 5'end from *EcoRI/NotI* subcloned cDNA fragment from the 10/3a clone.

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    1GATTCATGGGAGACCCCGACCGAGATGAAAACCCTGGGCGAAGAGTTTNCA
 51CGGAAAAACCAAGCGCTTTTTGTCCTGTGAGAACCCCGTTTAATAAGATA
 101AACTGAGTGGAGATGGAATTCACCCGCCTAAACTCGCGTTAAGAAATATG
 151ACAGGGGGAGCTGACTTTGAAACGAATAGTGCAAATGGAGAAAATTTTGA
 201GCTACCCAAGGCAAGTGAGAGGACATGACATTATGATGATGAGAATTGAA
 251AGACCCACTTTGGTACCTTTAAAGTTGGTGGAAATATTGAGCTGTTACAT
 301GGCCGTGATCCCAATCTTGTTCTTTCTTGTTGTGATGGCTCGAAATAGAT
 351CAATGTATAGGGGGAAAGTACTTAGATGACATCCACGCCTTACCAATGTT
 401AATAGATAAATATCCTTTTTTTTCCCCAAAAAAAAAAAAAAAAAAAAAGC
    451GGCCGC
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Figure 4. Nucleotide sequence of the 3'end from *EcoRI/NotI* subcloned cDNA fragment from the 10/3a clone.

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