Planta (1996) 199: 515–519

Planta

Disruption of an overlapping E-box/ABRE motif abolished high transcription of the *napA* storage-protein promoter in transgenic *Brassica napus* seeds

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Received: 20 November 1995/Accepted: 26 January 1996

Abstract. The storage protein napin is one of the major protein components of *Brassica napus* L. (oilseed rape) seeds. To investigate the transcriptional regulation of the napin promoter, different constructs of the napin gene *napA* promoter were fused to the *Escherichia coli uidA* gene and transformed into *B. napus*. A −152-bp promoter construct directed a strong expression of the marker gene in mature seeds. The 5′ deletion of an additional 8 completely abolished this activity. This deletion disrupted sequence motifs that are similar to an E-box, (**CA↓NNTG**) and an ABRE (**CGCCA↓CGTGTCC**) element (identity is indicated by bold face). Further, internal deletion of a segment corresponding to −133 to −121 caused an eightfold reduction in the activity of the −152 construct. This region contains an element, CAAACAC, conserved in many storage-protein gene promoters. These results imply that the E-box/ABRE-like sequence is a major motif of the *napA* promoter and suggest that the CAAACAC sequence is important for high activity of the *napA* promoter. Similar results have been obtained by analysing some of the constructs in transgenic tobacco, suggesting that many of the *cis*-elements in the *napA* promoter are conserved, at least in dicotyledonous species.

Key words: *Brassica* – Deletion analysis – Promoter – Seed – Storage protein

Introduction

The seed storage proteins are a diverse family of proteins exclusively expressed in the plant seed and serving as a carbon and nitrogen source during germination until the plant has the capacity to perform photosynthesis. Some spatial differences in the distribution of storage proteins exist in different plant species, but the three main tissues of the mature seed expressing storage proteins are the endosperm, the embryo and the aleurone layer.

The production of transgenic plants by use of *Agrobacterium*-mediated transformation has unravelled the transcriptional regulation of many plant genes. In particular, the seed storage-protein promoters have been studied and some elements known to be involved in storage-protein gene regulation have been identified (for reviews: Thomas 1993; Morton et al. 1995).

Plants with phenotypic mutations of importance for the seed-specific transcription have been identified and the corresponding genes have been cloned. For example, the viviparous mutant *Vp1* of *Zea mays* (McCarty et al. 1991) has been shown to be depleted of the storage protein Glb1 (Kriz et al. 1990) and similarly, the homologous *abi3* mutant of *Arabidopsis thaliana* has a diminished amount of 12S and 2S seed storage proteins (Finkelstein and Somerville 1990; Parcy et al. 1994). These two mutants are insensitive at certain developmental stages to the phytohormone cis-abscisic acid (ABA; Koorneef et al. 1984; McCarty et al. 1991). The precise role of the proteins affected by the mutation is unknown, but the seed-specific abi3 product can activate transcription of the 2S storage protein in the presence of ABA when constitutively expressed in vegetative tissues (Parcy et al. 1994). Another mutant in maize, *opaque2*, was found to differentially affect synthesis of the zein storage protein (Kodrzycki et al. 1989). This mutant gene was cloned and shown to encode a transcription factor belonging to the family of leucine zippers (bZIP; Schmidt et al. 1987). Another example is the *fus3* mutant of *Arabidopsis*, which also displays reduced amounts or almost absence of the 2S and 12S storage proteins in the seed. The phenotype of the *fus3* mutant is much the same as the *abi3* mutant, but the germination of seedlings is not insensitive to ABA (Bäumlein et al. 1994).

The characterisation of the proteins defined by these mutations and their role in transcriptional regulation will

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Abbreviations: ABA = abscisic acid; GUS = glucuronidase

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help to shed light on which *cis*-element in the promoter region responds to which regulatory pathway. To be able to couple these processes, detailed maps of the storage-protein promoter motifs have to be developed. We have therefore initiated a systematic study of the promoter of the *B. napus* 2S storage protein napin (Gustavsson et al. 1991; Stålberg et al. 1993). *Brassica napus* and *A. thaliana* 2S storage proteins are encoded by small gene families (Josefsson et al. 1987; Krebbers et al. 1988) and members of these gene families have been shown to be differentially transcribed (Guerche et al. 1990; Blundy et al. 1991). During seed development, napin starts to accumulate in the embryo axis. Somewhat later, napin can be found in the outer cotyledon and still later in the inner cotyledon (Höglund et al. 1991). Heterologous expression of successive 5′ deletion mutants of the napin *napA* promoter in transgenic tobacco showed that a construct with its 5′ end at − 309 directed a 74-fold higher expression than a fragment with its 5′ end at − 152. Further deletion to − 126 resulted in background level of the expression (Stålberg et al. 1993). Here, we show that the homologous expression of the *napA* promoter in transgenic *B. napus* seeds requires 8 base pairs (bp) for high activity from its minimal promoter coupled to the reporter gene β-glucuronidase (GUS).

Materials and methods

Plasmid construction and plant transformation. The *napA* promoter constructs have recently been described (Stålberg et al. 1993; own data not shown). The − 1101 and the − 309 constructs have been re-sequenced and an A to G mutation was observed in the leader sequence at + 16. Transformation of *B. napus*, cv. Westar (certified, provided by Svalöf Weibull AB, Svalöv, Sweden), hypocotyls was performed as described by De Block et al. (1989). Kanamycin-resistant plants were put on soil and grown in a greenhouse. Before flowering they were bagged to avoid cross-pollination. Seeds and leaves were harvested and frozen in liquid nitrogen. Deoxyribonucleic acid was prepared from leaves as described by Mettler (1987). The DNA was digested with *Hind* III, since a single *Hind* III restriction site is located 5′ to the *uidA* gene in the T-DNA. Southern blot analysis was carried out according to Sambrook et al. (1989) with a 2.13-kb *uidA* gene fragment as the probe.

β-Glucuronidase analysis. β-Glucuronidase activity was assayed by fluorometry on extracts obtained by homogenisation of ten seeds or approximately 10 mg of leaf tissue in GUS extraction buffer (Jefferson 1987). Homogenisation was performed on ice in Eppendorf tubes with a nylon pestle. The protein concentration of plant extracts was determined by the dye binding method (Bradford 1976). β-Glucuronidase assays were carried out as previously described (Stålberg et al. 1993) except that the data given were the average from two independent measurements.

Histochemical staining for GUS activity was carried out as follows: dry mature seeds from at least two independent transformants of each construct were incubated for 0.5 h in an 0.05 M phosphate buffer (pH 7.0), whereafter they were divided with a scalpel blade. The seed halves were then infiltrated in staining buffer containing 0.05 M sodium phosphate buffer (pH 7.0), 1.0 mM X-GlcA (5-bromo-4-chloro-3-indolyl glucuronide), 1 mM β-mercaptoethanol, 0.5 mM potassium ferricyanide and 0.5 mM potassium ferrocyanide, and incubated for 1 h at room temperature in vacuum. Histochemical stainings were then continued in the same buffer for 2 h at 37°C with agitation. The tissues were fixed/bleached in 75% methanol, 25% acetic acid for 1 h at 60°C. Sections of the stained seed coats were made by thin cuts by hand with a scalpel blade. The tissues were stored in water until photographed.

Results

High expression of napA promoter deletions in mature seeds. Transgenic *B. napus* plants were produced for the analysis of the expression of different portions of the *napA* promoter fused to the *uidA* reporter gene (Fig. 1). The activity in mature seed directed by the − 1101 deletion was approximately fivefold higher than that of the − 152 construct, while the activity of the − 309 deletion was about fourfold as high as the − 152 construct activity (Fig. 2). The deletion of 8 bp from − 152 to − 144 completely abolished the expression (Fig. 2). The deletion removed the sequence CTTCGCCA from the − 152 construct which caused the disruption of an E-box/ABRE-like sequence motif (Fig. 1), which suggests that this element is a crucial element for the transcriptional regulation of the *napA* gene. This E-box/ABRE element is in close context and partially overlaps with a region which shares some sequence identity with the motifs ESBF-II and opaque2 (Fig. 1). Alignment of the promoter region of 13 2S storage-protein genes shows that only the nucleotide at position − 145 is conserved upstream of the − 144 deletion and that the most conserved region is downstream of this position (Fig. 3).

An internal deletion − 152 d (Fig. 1), in which the region − 133 to − 121 was removed, significantly

Fig. 1. *Upper part*: Sequence of the *napA* promoter region from − 309 to the ATG translation initiation codon. End points of 5′ and internal deletions are indicated by *arrows*. *CAP*, transcription initiation site of the *napA* gene. Motifs discussed in the text are indicated by *underlining* (RY-repeat, CACGTG palindrome, RY-repeat, TATA-box). *Lower part*: Sequence of the region − 152 to − 114. End points of 5′ and internal deletion mutants are indicated above the sequence. Nucleotides matching the *cis*-elements below are indicated by *bold face*. *1*, opaque2 (Yunes et al. 1994); *2*, ABRE (Guiltinan et al. 1990); *3*, ESCBF-II (Hammond-Kosack et al. 1993); *4*, CAAACAC (an abundant seed storage-protein element, K. Stålberg, data not shown); *5*, flower-specific myb, consensus: A/C ACCT/A A A/CC, (Sablowski et al. 1994)

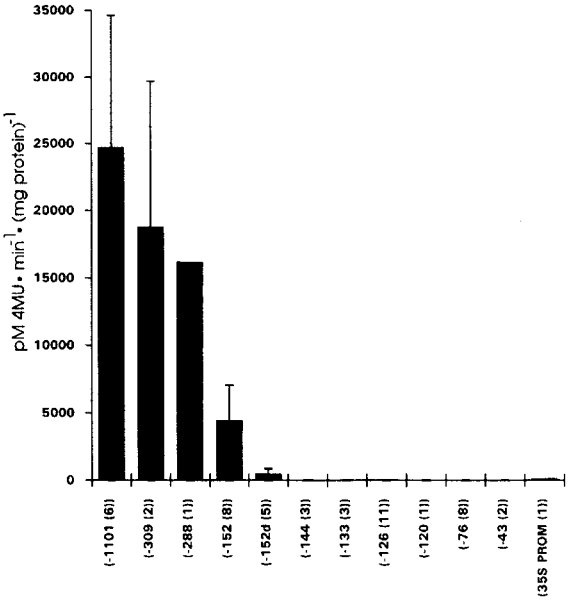

Fig. 2. β-Glucuronidase activity in mature seeds from transformed *B. napus* plants. Student's *t*-test indicates significant differences between the means of GUS activity directed by the following constructs: − 1101/152**, − 152/ − 152d***, − 152/ − 144***, 152d/ − 144*. Numbers in paranthesis indicate the number of transformed plants analyzed. *4MU*, 4-methyl-umbelliferone; * = *P*(0.1), ** = *P*(0.05) and *** = *P*(0.01) significance level

decreased the transcriptional activity from a − 152 construct (Fig. 2). The reason for this might be that the spacing of certain *cis*-elements was altered which impaired the binding of their corresponding transcription factors. However, we suggest that the deletion of this region removes an important motif, CTT**CAAACAC**CTA. Part of the sequence deleted is conserved in the upstream sequence of many seed storage-protein genes (Fig. 3). Furthermore, out of 113 promoters from prolamins, glutelins, albumins and globulin storage-protein genes, at least 103 have a 6-bp match to the motif CAAACAC (data not shown). Besides the − 144 deletion, the *napA* promoter constructs 5′-truncated at − 133, − 126, − 120, − 76 and − 43 did not exhibit an activity above background level (Fig. 2). This was surprising, since the internal deletion at − 133 to − 121 significantly decreased the activity and the constructs − 144 and − 133 containing this region were expected to direct a measurable activity in the seed.

Spatial expression directed from the napA constructs. In the mature seed, the spatial distribution of the *napA*-directed expression as detected by GUS histochemical staining was identical for the − 1101, − 309, − 152 and − 152 d constructs (Fig. 4). However, the expression from the shorter constructs was detectably lower. The aleurone layer inside the seed coat (Fig. 4) had a distinct activity, probably at least as high as the cells in the embryo. No GUS activity could be detected in seeds from plants transformed with the − 144 construct. None of the plants displayed a significant activity in the leaves (data not shown). However, it cannot be excluded that some tissues might display *napA*-directed GUS activity under certain growth conditions.

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                ↓-152   ↓-144                          ↓-133  ↓-126
BNANAPA         CTTCGCCACTTGTCACT----------------CCCTTCAAACACCTAA
BNANAPIN        CTTCGCCACTTGTCACT----------------CCCTTCAAACACCTAA
BJ2SSTP         CTTCGCCACTTGTCACT----------------CCCTTCAAACACCTAA
BO2SSPR         CTTCGCCACTTGTCCCT----------------CCCTTCAAACACCTAA
BNANAPB         GTTCGTCACTTGTCACT----------------CCCTTCAAACACCTAA
AT2SALBGB       GTTCGTCACTTGTCACT----------------CTCTTCCAACACCTAA
AT2SALBGA       GTTCGTCACTTGTCACT----------------CTTTTCCAACACATAA
AT2SALBGA       GTTCACGACTTGTCACTCCTCCAA----TTTGAGT---TTCGAACACCTAA
BNANAP1         GCGGA-GACTTGTCACTCCTCCA---CATTTGAGT---TTCCAACACCTAA
BNSSPC          GCGGACGATTTGTCACT----------------CACTTCAAACACCTAA
BNNAPINC        GCGGACGATTTGTCACT----------------CACTTCAAACACCTAA
BCNAPINB        GCG----ATTTGTCACT----------------CACTTCAAACACCTAA
AT2SALBGB       GCGGAA-ACTTGTCACT--TCCTTTACATTTGAGT---TTCCAACACCTAA

Core:             cAcTTGTCaCT                      TTCaAACACcTAA
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Fig. 3. Alignment of a segment of thirteen 2S seed storage-proteins promoters. The numbers indicated are from the 5′ end. BNANAPA (950–982, J02798, X58142), BNANAPIN (932–964, X14492), BJ2SSTP (947–979, X67833), BO2SSPR (947–979, X70333), BNANAPB (324–356), AT2SALBGB (2774–2806, Z24745), AT2SALBGA (160–192, Z24744), AT2SALBGA (1919–1962, Z24744), BNANAP1 (770–814, X17542), BNSSPC (116–148, J02782), BNNAPINC (2005–2037, M64633), BCNAPINB (1979–2008, M64632), AT2SALBGB (750–794, Z24745). Conserved nucleotides are shown at the bottom

Discussion

Evolutionarily conserved motifs are found in the napA promoter. A *napA* promoter fragment of 196 bp with its 5′ end at − 152 was found to direct a strong expression in mature *B. napus* seeds and to be inactive in leaves. A shortening of the fragment to − 144 completely abolished the activity. Either the ABRE-like or the E-box motif disrupted by this 8-bp deletion might be essential for the activation of the *napA* promoter. The ABRE elements are known to be involved in the ABA-dependent induction of other genes and have been shown to bind G-box-binding proteins belonging to the bZIP family of transcription factors (Giraudat et al. 1994). The fact that napin genes are induced by ABA indicates a function of the ABRE-like motif in the regulation of the *napA* gene (DeLisle and Crouch 1989). It is intriguing that this motif lacks an ACGT core sequence, which has been suggested to be of importance for high-affinity binding of G-box-binding proteins (Izawa et al. 1993), which argues against a regulatory role for this element. On the other hand, it has been shown that the transcription factor opaque2, which is a bZIP protein, binds with high affinity to a sequence lacking an ACGT core (de Pater et al. 1994). Furthermore, several G-box-binding proteins exist, which can hetero- and homodimerise (Schindler et al. 1992; Pysh et al. 1993), which suggests that *cis*-elements with similar but not identical sequences can be the target for these transcriptional complexes. Another putative *cis*-element, whose disruption might account for the loss of activity caused by the deletion of the 8 bp to − 144, is the E-box (CANNTG) motif. In the β-phaseolin gene promoter, E-box motifs located at different positions act synergistically in the regulation of the gene (Kawagoe et al. 1994). The area of the *napA* promoter around − 152 to − 132 also displays some similarity to the binding sequences of two other transcription factors, opaque2 and ESCBF-II (Hammond-Kosack et al. 1993; Yunes et al. 1994). The opaque2 motif is the DNAse-protected binding site on the

Fig. 4. Histochemical GUS staining of mature seeds from transgenic *B. napus* plants. From top to bottom, seeds from plants transformed with − 1101, − 309, − 152 − 152d and − 144 *napA* deletion constructs. *Left panel*: sections of mature seeds. *Right panel*: transverse sections of the seed coat. Axis (*A*), cotyledons (*C*), aleurone (*AL*) and seed coat (*SC*) are indicated. Left panel bar = 500 μm; right panel bar = 30 μm

upper strand of the α-prolamin promoter. The *napA* sequence displays 11 identities out of 21 positions to that site. The identity to the ESCBF-II site is 7 out of 10 bp for the *napA* sequence. The relevance of the *napA* promoter similarity to these two sites might be disputable, since genes regulated via these sequences are mainly expressed in the aleurone and endosperm of transgenic dicotyledonous plants.

Further downstream of these sites, in the region between − 130 to − 119, the *napA* promoter shows some similarity to the binding site of a myb transcription factor and to the conserved CAAACAC element. The myb factor is involved in the regulation of phenylpropanoid synthesis in *Anthirrinum majus* (Sablowski et al. 1994). The importance of this region for the *napA* promoter regulation was demonstrated by the eightfold reduction in activity when this region was deleted. Furthermore, the sequence from − 144 to − 123 of the *napA* promoter was previously shown to bind nuclear proteins in an electrophoretic-mobility-shift assay (Gustavsson et al. 1991) and similar regions of the Brazil nut 2S storage protein promoter are protected in an DNAse I footprint assay (Grossi de Sa et al. 1994).

Two motifs downstream from the CAAACAC homologous motif that do not alone in their context have any activity are the RY repeats and the CACGTG palindrome (Fig. 1). It is well known that the RY repeat plays an important role in the regulation of other storage-protein promoters (Morton et al. 1995). The role of the RY repeat is reminiscent of the results obtained by the deletion of the CAAACAC element demonstrated in this paper. Several deletions (− 144, − 133, − 126, − 120, and − 76) containing the CACGTG palindrome located at − 66 to − 61 did not activate embryonic transcription in its *napA* promoter context. This was surprising, since it has been demonstrated that the CACGTG palindrome in the β-phaseolin promoter is of importance for the regulation of this gene. The mutation of this motif in the β-phaseolin promoter caused a significant decrease in the activity, and mutation of both the palindrome and two E-boxes was shown to drastically decrease the activity (Kawagoe et al. 1994). Whether the CACGTG palindrome is of importance for the tissue-specific expression of the *napA* promoter remains to be elucidated.

Heterologous expression of napA promoter constructs in tobacco reflects the main features of their expression in B. napus. The use of a heterologous system to produce stable transformants is in some cases a necessity for studies of promoters isolated from plant species difficult to transform. However, heterologous systems do not always show an expression consistent with that of the homologous plants. One example is the expression driven by the *opaque2* promoter, which in *Z. mays* is restricted to the endosperm of the seed. When the activity of the *opaque2* promoter was studied in transgenic tobacco, it was found to direct expression both in the endosperm and in the embryo (Gallusci et al. 1994). *Brassica napus* is obviously possible to transform. However, for the production and maintenance of the large number of transgenic plants necessary in promoter studies, it is much more laborious and time consuming to transform *B. napus* than tobacco. For the initial screening of the *napA* promoter segments, we therefore transformed the various *napA* promoter constructs into tobacco (Stålberg et al. 1993).

Comparison of the spatial distribution of *napA* expression in transgenic tobacco (Stålberg et al. 1993) and *B. napus*, showed both differences and similarities. For both types of transgenic plant, the main activity was located to the embryo. Since the mature *B. napus* seed, in contrast to the tobacco seed, only has a tiny endosperm, the activity in this tissue of the two species could not be compared. However, the *napA* promoter is active in the aleurone layer in *B. napus* (Fig. 4). A similar cell layer which is situated between the seed coat and the endosperm in tobacco also displayed a marked *napA* transcriptional activity (Stålberg et al. 1993). On the other hand, some definite differences exist between the transcription directed by the *napA* promoter in *B. napus* and in tobacco. The − 1102 promoter construct was 12-fold less active in transgenic tobacco seeds than in *B. napus* seeds and the − 309 promoter constructs seems to be 4-fold less active.

Even more important, the − 152 promoter construct had a 65-fold higher activity in transgenic *B. napus* seeds than in tobacco seeds. Disregarding these quantitative differences, the activity of the *napA* promoter in tobacco mirrors its activity in *B. napus* (Stålberg et al. 1993), suggesting that many *cis*-elements in the *napA* promoter are functionally conserved, at least in dicotyledonous species. More-detailed studies might, however, also unravel qualitative differences between the two expression systems.

Dr. Enno Krebbers, (Plant Genetic Systems N.V., Gent, Belgium) kindly provided the *Agrobacterium* strain C58C1(PMP90). Bertil Blom and Elisabeth Westergren are gratefully acknowledged for expert technical assistance. This work was supported by grants form the Swedish Natural Science Research Council, The Swedish Research Council for Forestry and Agriculture, The Swedish Institute and the Nilsson-Ehle Foundation.

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