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Abscisic acid does not influence the subcellular distribution of the HYL1 protein from *Arabidopsis thaliana*★

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HYL1 is a nuclear protein involved in the processing of miRNAs but its exact function remains unknown. *Arabidopsis thaliana hyl1* mutants exhibit hypersensitivity to ABA. We decided to answer the question whether ABA affects the HYL1 protein localization within the cell and show that it does not. We also studied the expression of HYL1 in different tissues and organs. In this paper we show for the first time the expression profile of the HYL1 protein using anti-HYL1 antibodies. The protein is present in seedlings and mature plants in all organs studied, with the highest amount in inflorescences. *A. thaliana* HYL1 protein has several repetitions of a 28-amino-acid sequence at the C-terminus that confer protein instability. Our bioinformatic analysis of HYL1 homologs in different *Brassica* species shows that this repetition is typical only for *Arabidopsis*. This may suggest a relatively late evolutionary acquisition of the C-terminal domain.

Keywords: *Arabidopsis thaliana*, HYL1 protein, abscisic acid, subcellular distribution, expression profile

INTRODUCTION

The *Arabidopsis HYL1* gene encodes a nuclear double-stranded RNA-binding protein involved in microRNA (miRNA) biogenesis. The *hyl1* null mutant, designated *hyponastic leaves 1*, exhibits severe developmental defects, altered hormone response and reduced miRNAs level (Lu & Fedoroff, 2000; Han *et al*., 2004; Vazquez *et al*., 2004). The HYL1 protein is a part of a large (300–500 kDa), rather unstable complex (Song *et al*., 2007). It is suggested that DCL1 is also a component of the HYL1 complex and the interaction of HYL1 with DCL1 is important for the efficient and precise processing of at least some miRNA primary transcripts during plant miRNA biogenesis (Kurihara *et al*., 2006). Recent studies have shown that another protein, SERRATE (SE), is also involved in the accumulation of miRNA and contributes to normal plant development along with DCL1 and HYL1 (Yang *et al*., 2006). Results of bimolecular fluorescence complementation (BiFC) and fluorescence resonance energy transfer (FRET) experiments show that HYL1, DCL1 and SE interact in bodies located in the nucleus. It is likely that the DCL1–HYL1–SE nuclear bodies are involved in miRNA production since miRNA precursors are localized within these structures. Two protein markers of Cajal bodies, SmD3 and SmB, co-localize with HYL1, DCL1 and SE, but the DCL1–HYL1–SE complexes do not localize in the bodies identified by another marker of Cajal bodies — coilin (Fang & Spector, 2007; Fujioka *et al*., 2007; Song *et al*., 2007). It has

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Abbreviations: ABA, abscisic acid; DCL1, dicer like 1; DRB, dsRNA-binding; dsRBM, dsRNA-binding motif; dsRBD, dsRNA-binding domain; dsRNA, double-stranded RNA; GFP, green fluorescent protein; miRNA, microRNA; HYL1, hyponastic leaves 1; NLS, nuclear localization signal; SE, serrate.

already been suggested that SmD3-containing Cajal bodies take part in the processing of transcripts during formation of the *A. thaliana* RISC complex (Li *et al.*, 2006).

It has been shown that at least several microRNAs play a role in the plant response to abiotic and biotic stresses (Sunkar *et al*., 2007). Tolerance to specific stresses is mediated mainly by the plant hormone abscisic acid (ABA) (Xiong *et al*., 2002). The *A. thaliana hyl1* mutant exhibits hypersensitivity to ABA. Since it also shows impairment of the microRNA biogenesis, we decided to look for the possibility of an involvement of ABA in HYL1 subcellular localization. In this paper we show that ABA has no influence on the HYL1 localization in the cell. Western blot analysis of the HYL1 expression profile has revealed the presence of this protein in all plant organs with the highest amount present in the inflorescence, in agreement with earlier *HYL1* mRNA expression data (Han *et al*., 2004).

MATERIALS AND METHODS:

Plants. All experiments were performed on *Arabidopsis thaliana* ecotype Columbia-0, growing at 22°C at 12-h light, 12-h dark photoperiod. *Nicotiana tabacum* var. *xanthi* plants were grown at 22°C at 16-h light, 8-h dark photoperiod.

Reagents. Anti-HYL1 rabbit polyclonal antibody was raised (Agrisera, Sweden) against the 15 aa C-terminal peptide (N-CMSGLKEAAFGSVET-C) predicted to be highly specific to HYL1 as described in (Han *et al*., 2004). Starlight™ Chemiluminescent Substrate and luminescence enhancer Opti-Membrane™ Reagent were from MP Biomedicals Inc. (Ohio, USA). TRIzol Reagent used for RNA isolation, as well as all materials for insect cell growth and transformation were from Invitrogen Life Technologies. The Talon affinity matrix for purification of His_6-tagged proteins was from Clontech BD Biosciences. PVDF membranes were from Millipore or Amersham Biosciences. pGEM-T Easy and reverse transcriptase were purchased from Promega. Cellulase and macerozyme R-10 were from Serva; drisellase and abscisic acid were from Sigma Aldrich. If not stated otherwise, enzymes were from Fermentas.

cDNA isolation from *A. thaliana*. Total RNA was extracted from mature leaves of *hyl1* mutants and wild type plants using TRIzol Reagent, and the first strand of cDNA was synthesized using MMLV reverse transcriptase according to the manufacturer's protocol. The sequences of *HYL1* cDNA — specific primers were as follows: 5′-CTGGTCGACAATGACCTCCACTGATGTTTC-3′ (forward) and 5′-GGATCCTGCGTGGCTTGCTTCTGTC-3′ (reverse). After amplification the cDNA was cloned into the pGEM-T Easy vector.

Western blot analysis. Total protein extract was obtained from mature leaves of *A. thaliana* wild type plants and homozygous *hyl1* T-DNA insertion mutants (SALK_064863). For Western blotting analysis, 10 μg of total protein extracts was loaded on a 10% polyacrylamide gel. For HYL1 expression profile analysis, tissues were collected from 6-day-old seedlings, 32-day-old roots, 32-day-old rosette leaves, 37-day-old stem leaves and inflorescences. The proteins were subjected to electrophoresis and electrotransferred to PVDF Immobilon-P membrane. The membranes were blocked overnight at 4ºC with 3% BSA (bovine serum albumin) solution in TBS-T (20 mM Tris/HCl, 137 mM NaCl, 0.1% Tween 20) buffer, and subsequently incubated with anti-HYL1 antibodies and with anti-rabbit IgG antibodies conjugated with alkaline phosphatase. The targeted protein bands were visualized by chemiluminescence using Starlight™ Chemiluminescent Substrate and luminescence enhancer Opti-Membrane™ Reagent.

HYL1 immunoprecipitation from *A. thaliana* protein extract. In a standard immunoprecipitation reaction, 0.5 g of *Arabidopsis* leaves was ground into fine powder under liquid nitrogen and then homogenized in 1 ml of extraction buffer pH 7.5 (50 mM Tris/HCl, 50 mM NaCl, 0.1% Triton-X) containing protease inhibitor cocktail (1 tablet/10 ml; Roche). Cell debris was removed by centrifugation at 4°C (22 000 × *g*, 20 min). The supernatant was collected and protein concentration was determined using Bio-Rad Protein Assay (Bio-Rad). Protein concentrations were adjusted to 10 mg/ml with extraction buffer and 0.5 ml of extract was precleared by incubation with 50 μl of Protein A Sepharose (Amerham Biosciences) at 4°C for 30 min. Precleared extracts were then incubated with 20 μl of anti-HYL1 antibodies and 30 μl of Protein A Sepharose at 4°C for 2 h. Immunoprecipitates were washed three times (20 min. each) in a washing buffer pH 7.5 (50 mM Tris/HCl, 150 mM NaCl, 0.1% Triton-X).

Protoplast transfection and localization studies. The cloned *HYL1* cDNA was inserted into the pDEDH72-eGFP vector (a kind gift from W. Filipowicz) at the N-terminal end of the GFP-coding sequence by using the *Sal*I and *Bam*HI sites. The *HYL1-GFP* chimeric gene was expressed from the cauliflower mosaic virus 35S promoter. Isolation, purification and transient transfection of *A. thaliana* and *N. tabacum* protoplasts were carried out as described by (Prols *et al*., 1988; Li *et al*., 2002), respectively.

Protoplasts were transfected with recombined pDEDH72-HYL1-eGFP plasmid. PEG-mediated transfection was applied to introduce plasmids into the protoplasts of *A. thaliana* and *N. tabacum* var. *xanti*. Thirty micrograms of plasmid DNA was used for single transfection. After 24 h of incubation at room temperature, transient expression of HYL1-GFP was

observed in a confocal Zeiss LSM5 10 microscope using a plan-apochromat 63×/1.4 Oil DIC objective. The argon laser 477 nm line and the helium-neon laser 543 nm line were used for the fluorescence excitation and detection, respectively. In the case of the influence of ABA on the subcellular localization of HYL1, 50 μM ABA solution was prepared in 0.02 M NaOH and added to the protoplast suspension. Subsequently, protoplasts were incubated for 20, 45 or 180 min and the localization of the fused protein was observed.

Expression of HYL1 protein in baculovirus system. Recombinant protein isolation and purification. The *HYL1* cDNA was cloned from the pGEM-T Easy vector into the *Bam*HI and *Eco*RI sites of the pFAST-Bac HT B vector (Invitrogen). All procedures were conducted as described in the Invitrogen Life Technologies Instruction Manual. The yield of the HYL1 protein in the baculovirus Bac-to-Bac system was 1.5–2 mg/liter of medium.

Protein disorder prediction. Protein disorder was predicted using the VL-XT Predictor (Romero *et al*., 1997; 2001; Li *et al*., 1999) at PONDR (Predictor of Natural Disordered Regions; www.pondr.com) server. Domains in HYL1 were assigned according to (St Johnston *et al*., 1992).

Homolog searches and analysis. In order to find homologs of the HYL1 protein (NP_563950.1), the pBLAST algorithm (Altschul *et al*., 1997; Schäffer *et al*., 2001) was used to search the non-redundant version of the current NCBI sequence databases. All homologous sequences from the *Brassicaceae* family were aligned using the T-coffee algorithm (Notredame *et al*., 2000). An illustration of the alignment was prepared with the Jalview program (Clamp *et al*., 2004).

RESULTS

HYL1 expression profile

To study the HYL1 expression profile at the protein level we decided to produce antibodies against HYL1 using a protein peptide fragment (401–415 aa), according to the procedure described by (Han *et al*., 2004). In our Western blot experiments, the rabbit antibodies raised against the HYL1 short fragment recognized specifically the HYL1 protein and another, unknown protein from wild type plants. The same analysis with a protein extract prepared from the T-DNA insertion mutant of the *HYL1* gene showed no signal at the level of the HYL1 protein, however, the unknown protein was still detected (Fig. 1A).

To confirm the presence of the HYL1 protein during different stages of plant development and in different organs, we isolated total proteins from *A. thaliana* seedlings, roots, rosette, and stem leaves and inflorescences and subjected then to Western blotting. The HYL1 protein was present in young developing plants and in all organs of the mature ones, with the highest HYL1 expression level in the inflorescence (Fig. 1B).

To check whether the anti-HYL1 antibodies recognize the native structure of HYL1 we immunoprecipitated the HYL1 protein from protein extracts prepared from *Arabidopsis* wild type plants and from the *hyl1* mutant. Western blot analysis revealed the presence of the HYL1 protein on the blot containing precipitates from the wild type plant and its absence in the case of the *hyl1* mutant. This suggests that the HYL1 antibodies recognize the native form of the HYL1 protein and can be used for identification of other proteins in the HYL1 complex by co-immunoprecipitation (Fig. 1C). Immunoprecipitation of the HYL1 protein from the wild type and the *hyl1* mutant protein extracts did not give any signal in Western blot at the gel migration position of HYL1 in the control experiment when pre-immune rabbit serum was used (not shown).

The influence of abscisic acid on the subcellular localization of the HYL1 protein

The *A. thaliana hyl1* mutant shows hypersensitivity to abscisic acid resulting in a complete inhibition of seed germination in the presence of 0.4 μM

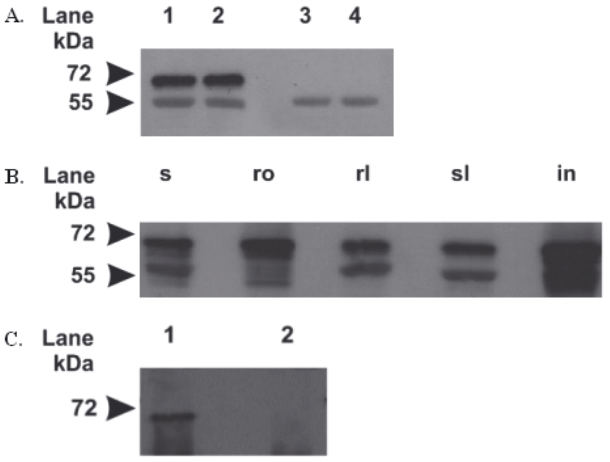

Figure 1. Western blot detection of HYL1 expression with anti-HYL1 antibody.
A. The HYL1 protein expressed in *Arabidopsis thaliana*; lanes 1 and 2, total protein isolated from rosette leaves of wild type plants, lanes 3 and 4, total protein isolated from rosette leaves of *hyl1* T-DNA insertion mutants. B. Total protein isolated from seedlings (s), roots (ro), rosette leaves (rl), stem leaves (sl) and inflorescences (in) of wild type plants. C. Immunoprecipitation of the HYL1 protein expressed in *A. thaliana* with anti-HYL1 antibody; lane 1, HYL1 protein immunoprecipitated from rosette leaves of wild type plants; Lane 2, HYL1 protein immunoprecipitated from rosette leaves of *hyl1* T-DNA insertion mutants.

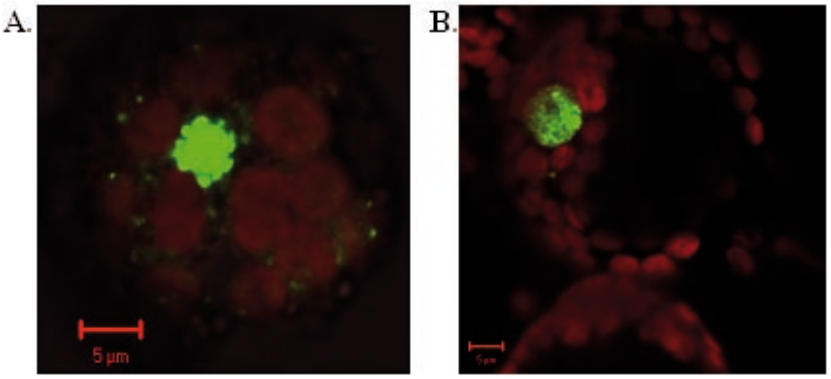

Figure 2. Transient expression of the HYL1-GFP fusion protein (green) in *A. thaliana* (A) and *N. tabacum* var. *xanthi* (B) mesophyll protoplasts.
Red fluorescence comes from chloroplasts. Because of strong chlorophyll autofluorescence, 477 nm line was used for excitation instead of 488 nm standard one. Further details are presented in Materials and Methods.

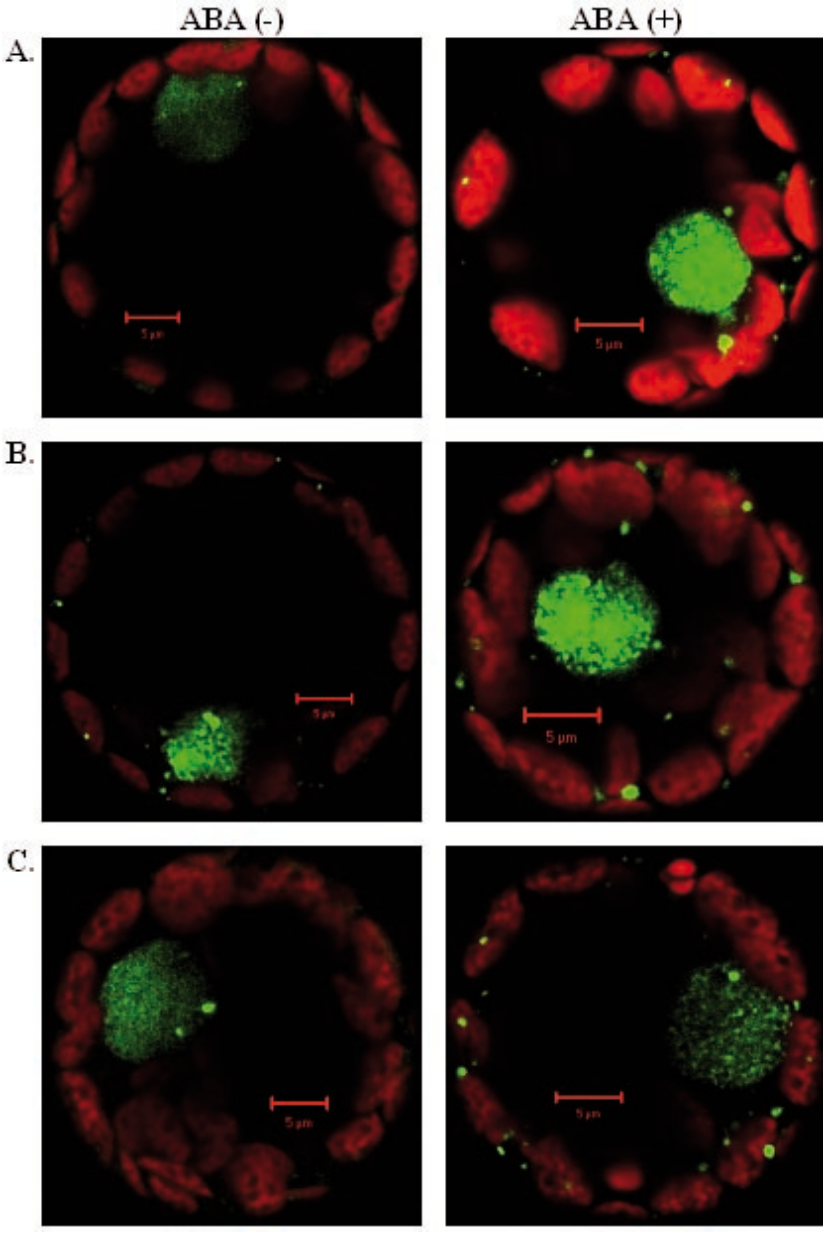

Figure 3. HYL1-GFP localization in tobacco protoplasts in the presence or absence of ABA.
(A), (B), and (C): transfected protoplasts after 20, 45, and 180 min of treatment with buffer (images on the left) or 50 µM ABA (images on the right).

ABA (not shown; Lu & Fedoroff, 2000). To answer how ABA is involved in the HYL1 action we decided to study the influence of this hormone on the subcellular localization of the HYL1 protein. First we performed transfections with a HYL1-GFP construct into *A. thaliana* and *N. tabacum* protoplasts. As expected, in *A. thaliana* the fusion protein was present in the nucleus and formed small, well-defined speckles. We obtained the same results when tobacco protoplasts were used (Fig. 2). Presumably as a result of the very strong overexpression of the fusion protein, some small green speckles were also present outside the nucleus. This experiment proved that both systems tested can be used to study the subcellular localization of the *A. thaliana* HYL1 protein. Since tobacco protoplasts are larger and easier to isolate and transfect, we decided to use them in further experiments. The protoplasts were transfected with the HYL1-GFP construct and incubated in darkness for 24 h. The protoplasts were divided into two groups: one was incubated in a medium containing 50 µM ABA, the second in a medium without the hormone. HYL1-GFP localization was observed in the protoplasts from both groups after 20, 45 or 180 min from ABA application. In all the cases we observed the same HYL1-GFP localization within the cell, namely, in multiple, well-defined bright

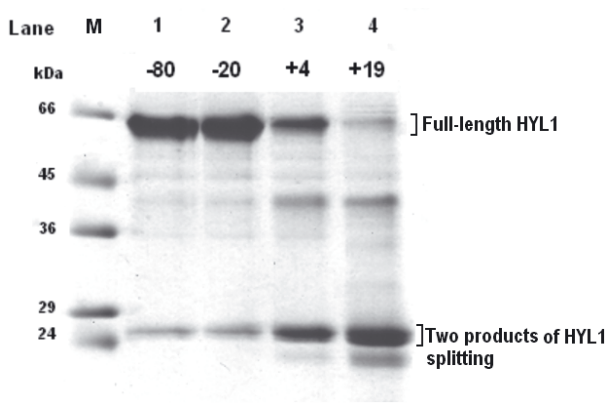

Figure 4. Stability of full-length HYL1 protein stored at different conditions for three weeks.
Polyacrylamide denaturing gel (15%) stained with Coomassie Brilliant Blue. Lanes: M, molecular mass marker; 1, protein sample stored at –80°C (control); 2, at –20°C; 3, at +4°C; 4; at +19°C in the following buffer: 20 mM Tris/Cl, pH 8.8, 200 mM NaCl and 250 mM imidazole.

spots in the nucleus (Fig. 3). Thus, ABA does not influence the subcellular distribution of the HYL1 protein.

HYL1 overexpression and stability

The efficiency of HYL1 overexpression in the baculovirus system was high: 1.5–2.0 mg/medium liter. Since the expression efficiency was good, we intended to use the protein for crystallization studies. However, we found that the protein is unstable. After three weeks of storage at +4°C about 50% of the protein was degraded, while storage at +19°C resulted in about 90% degradation (Fig. 4). The two major degradation products have almost the same molecular masses (about 24 kDa, Fig. 4) and are present in similar amounts. This would mean that the protein is likely to break down spontaneously into two parts: one containing the dsRBD domain and the other one containing the NLS signal and six 28-amino-acid repeats (as deduced from the HYL1 protein primary structure, see Fig. 5).

The HYL1 sequence was analyzed with PONDR to predict the ordered/disordered regions. The results show that the C-terminal part containing NLS and the 28-amino-acid repeats is intrinsically disordered (ID, unstructured), while the N-terminal part of the protein exhibits a highly ordered structure (Fig. 5). It is clearly visible that a dramatic change in the HYL1 structure takes place in the middle of the protein, and it can also explain the observed protein degradation into two parts.

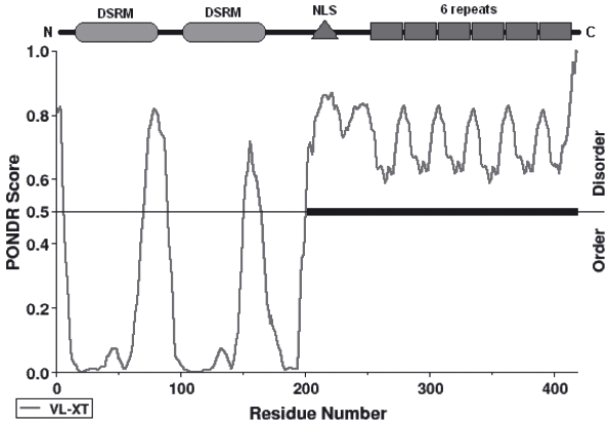

Figure 5. Analysis of the HYL1 structure (top) with PONDR prediction of ordered/disordered regions program (bottom).
Top: DSRM, putative dsRNA-binding motifs; NLS, putative bipartite nuclear localization sequence. Bottom: Each residue (*X*-axis) is assigned a disorder score (*Y*-axis) by the predictor based on the attributes of amino acids surrounding the residue. Predicted scores ≥ 0.5 indicate disorder.

DISCUSSION

The existence of microRNAs have been discovered as a consequence of intense work on the identification of short RNAs involved in RNA interference process (Szweykowska-Kulińska *et al*., 2003; Lesicka *et al*., 2004). HYL1 protein is involved in the maturation of pre-miRNA precursors. In this paper we have shown for the first time the expression profile of the HYL1 protein using anti-HYL1 antibodies. The protein is present in seedlings and mature plants in all the studied organs. However, it predominates in the inflorescence, which is in concordance with the mRNA expression profile data presented in the Arabidopsis eFP Browser (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). This may be explained by the involvement of many HYL1-dependent microRNAs in the generative processes.

We also present data concerning the subcellular localization of HYL1 in the presence or absence of ABA. It has been found that the RNA-binding protein AKIP1 from *Vicia faba* (interacting with the abscisic-acid-activated protein kinase AAPK), which is usually dispersed in the guard cell nuclei, redistributes and concentrates in subnuclear foci after ABA treatment (Li *et al*., 2002). The same has been reported for the RNA-binding UBA2a protein from *A. thaliana*, the closest homologue of the *V. faba* AKIP1 protein. It also reorganizes in the nucleus in "speckles" in response to ABA and is possibly involved in the regulation of RNA metabolism during ABA signaling (Riera *et al*., 2006). Since HYL1 is an RNA-binding protein and the *hyl1* mutant exhibits altered ABA response we hypothesized that ABA may change HYL1 subcellular localization. HYL1 has earlier been reported to be present in the nucle-

us in both dispersed and concentrated forms in subnuclear foci (Lu & Fedoroff, 2000). Our results show that ABA does not substantially influence the distribution of the HYL1-GFP protein in the cell. However, more concentrated HYL1-containing foci within the nucleus are observed in the case of ABA-treated protoplast (see Fig. 2). High-resolution images have to be obtained for an unambiguous statement about the possible changes in the subnuclear localization of HYL1-containig bodies.

Two hypotheses explaining how ABA is involved in the HYL1 action are possible: (i) HYL1 itself is a negative regulator of a putative intermediate that acts in the ABA signaling pathway or (ii) microRNA(s) that requires HYL1 for efficient maturation controls the level of the putative negative regulator in ABA signal transduction. In both cases an altered response to ABA will lead to hypersensitivity to this hormone, as it is observed in the *hyl1* mutant. We think the second hypothesis is more likely and we are preparing experiments to find all targets of microRNAs that require HYL1 protein for their biogenesis. Other *A. thaliana* mutants have been described that show hypersensitivity to ABA and in which the RNA metabolism is altered. These are: *abh1*, *cbp20, sad1*, and *sad2*. The *ABH1* and *CBP20* genes encode two cap-binding proteins — CBP80 and CBP20, respectively, and are involved in pre-mRNA splicing (Hugouvieux *et al.*, 2001). The SAD1 protein belongs to Sm-like snRNP proteins required for mRNA splicing, export, and degradation (Xiong *et al*., 2001). Localization of the SAD1 protein was studied in *Arabidopsis*. It was found in the nucleus and cytoplasm (Kufel J., Kruszka K., unpublished). The SAD2 protein, also mainly localized in the nucleus, belongs to the β-importin family and is involved in nuclear transport (Verslues *et al*., 2006). These observations suggest a role for nuclear proteins and nuclear transport in ABA signal transduction. However, there are no reports indicating redistribution of these proteins within the cell/nucleus when ABA is present. Thus the AKIP1/UBA2a proteins remain to be the only two proteins for which ABA-dependent localization has been observed.

The question about the exact function of the HYL1 protein remains unanswered. Analysis of the *hyl1* mutant showed that the protein is involved in the processing of several miRNAs, probably during the interactions with the main miRNA, processing enzyme in plants, DCL1 (Hiraguri *et al*., 2005). We have successfully overexpressed the HYL1 protein from *A. thaliana* in baculoviruses, however, our attempts to crystallize it have been so far unsuccessful since the protein spontaneously splits into two parts. The HYL1 protein belongs to the DRB protein family (dsRNA-binding) and has two dsRNA-binding domains (dsRBD1 and dsRBD2) in its N-terminal half, a bipartite nuclear localization motif and six repetitions of a 28-amino-acid sequence at the C-terminus. Only the dsRBD1 domain of HYL1 is essential for dsRNA binding *in vitro*, whereas dsRBD2 contributes to its protein–protein interactions (Hiraguri *et al*., 2005). It is presumed that the C-terminal repeats are also responsible for binding other unknown protein partners (Lu & Fedoroff, 2000). To answer the question why the HYL1 protein breaks up so rapidly, we used the PONDR program for protein disorder prediction. The results indicate that the protein could be divided into two parts: a highly structured N-terminal part containing the two dsRBDs domains and a highly unstructured C-terminal part containing the NLS signal and the six 28-amino-acid repeats. This could be the reason why the disordered C-terminus destabilizes the protein, resulting in its fragmentation into two parts (Dobson, 1999). The experiments were done *in vitro* using purified HYL1 protein. *In vivo* HYL1 may be stable since it functions in a complex of about 300–500 kDa (Han *et al*., 2004; Song *et al*., 2007) with at least one known protein (DCL1), which might stabilize the C-terminus and prevent HYL1 from degradation detected *in vitro*. What is the function of the HYL1 disordered part in addition to targeting the protein to the nucleus? It is known that intrinsically disordered proteins and regions may carry out numerous biological functions including cell signaling, molecular recognition, and protein modifications (including phosphorylation) (Iakoucheva *et al*., 2004; Radivojac *et al*., 2007). However, *hyl1* plants transformed with a *HYL1* cDNA fragment encoding only two N-terminal dsRBDs displayed a complete rescue of the phenotypes conferred by the *hyl1* mutation (Wu *et al*., 2007). Moreover, a comparison of these transgenic and wild type plants revealed that the dsRBDs of HYL1 restore the accumulation of miR165, miR160 and miR319, and guide the post-transcriptional silencing of the *REV* and *ARF17* genes. These findings indicate that the dsRBDs are essential for HYL1 function and the C-terminal NLS and six repeats are not required (Wu *et al*., 2007). This statement can be strengthened by our bioinformatic analysis of HYL1 homologs in different *Brassica* species: *A. thaliana* is the only species where the HYL1 protein has several repetitions of the 28-amino-acid sequence at the C-terminus. All other species only have a single sequence motif in this region (Fig. 6). Furthermore, even between *A. thaliana* ecotypes there are differences in the number of the repeated sequences. In the Wassilevskija, Landsberg erecta and Nossen ecotypes there are five repetitions of the 28-amino-acid motif, in contrast to the Columbia ecotype which has six (Han M.-H., personal communication). This observation may suggest a relatively late evolutionary acquisition of the C-terminal domain. The function of the HYL1 C-terminal part remains obscure.

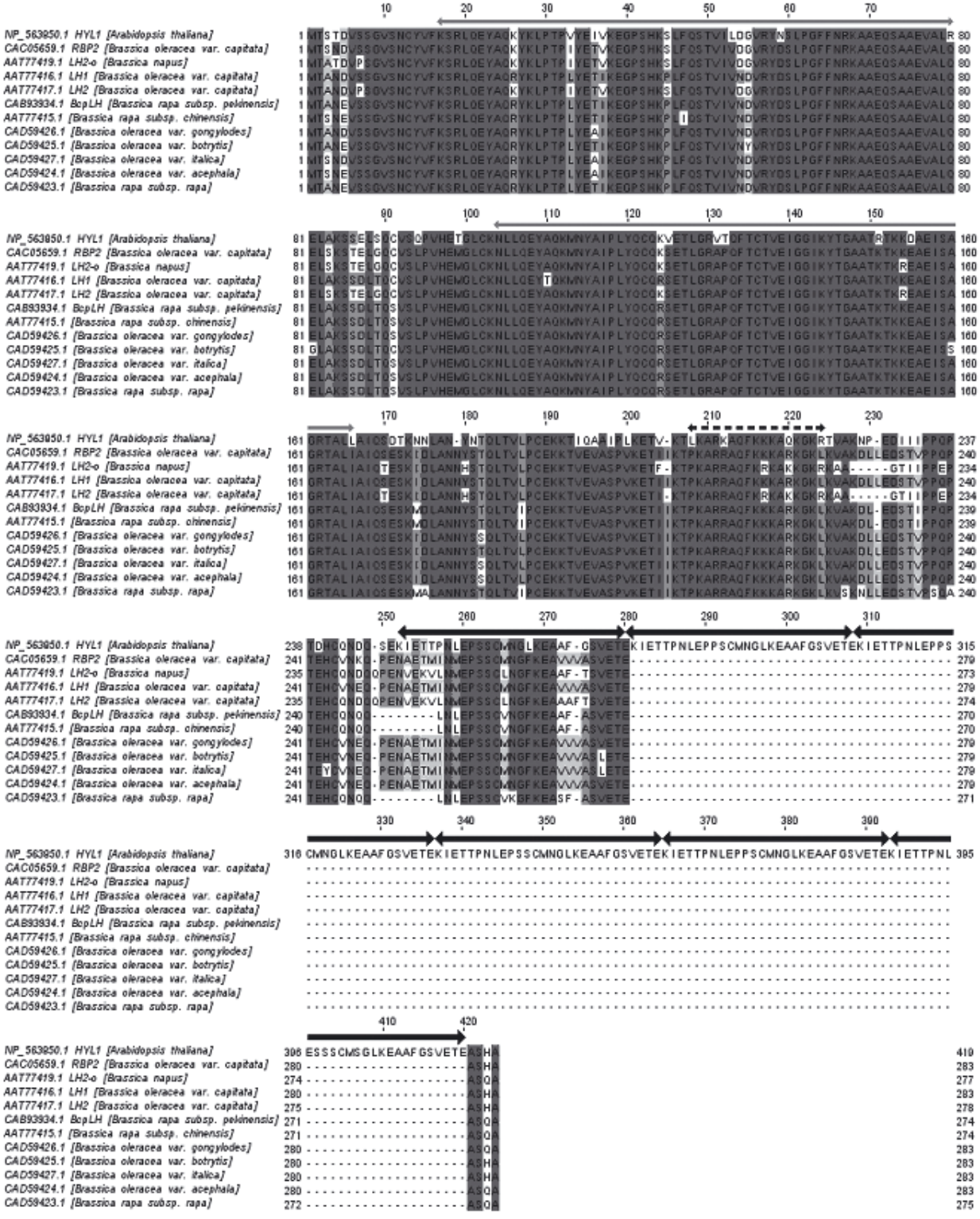

Figure 6. Alignment of the amino-acid sequences of HYL1 homologs from *A. thaliana* and *Brassica* species.
Black thick arrows above alignment indicate C-terminal repeats in HYL1 sequence.

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