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Recombinant Whirly1 translocates from transplastomic chloroplasts to the nucleus

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ABSTRACT

Whirly1 was shown to be dually located in chloroplasts and nucleus of the same cell. To investigate whether the protein translocates from chloroplasts to the nucleus, we inserted a construct encoding an HA-tagged Whirly1 into the plastid genome of tobacco. Although the tagged protein was synthesized in plastids, it was detected in nuclei. Dual location of the protein was confirmed by immunocytological analyses. These results indicate that the plastidial Whirly1 is translocated from the plastid to the nucleus where it affects expression of target genes such as *PR1*. Our results support a role of Whirly1 in plastid-nucleus communication.

1. Introduction

Whirly proteins belong to a small family present in all higher plants. Each species has at least two Whirly proteins. Originally, Whirly1 has been described as the transcriptional activator p24 of the *PR10a* gene of potato [1]. Later it has been shown that the sequence of Whirly1 has an N-terminal extension encoding a plastid targeting peptide enabling the import of the protein into chloroplasts [2]. Fluorescence of a Whirly1:GFP fusion protein was found to be associated with speckles resembling nucleoids [2]. Accordingly, Whirly1 has been identified as a protein of the transcriptionally active chromosome [3]. These findings are in accordance with the recently proposed function as an anti-recombination protein in plastids [4]. In chloroplasts of maize and barley Whirly1 has been detected in addition in the stroma where it binds to intron containing RNA species [5,6].

Immunological analyses with an antibody directed against the Whirly1 protein of barley revealed that Whirly1 is located in chloroplasts and the nucleus of the same cell [7]. Only few proteins were described to have a dual localisation in an organelle and the nucleus. One example is the mitochondrial pentatricopeptide repeat (PPR) protein localised to the nucleus and mitochondria (PNM1) [8]. Such proteins were proposed to be stored in the organelle and to be redirected to the nucleus in response to certain stimuli [9].

Targeting of dually located proteins might depend on the cell type or the developmental stage of the plant. For barley Whirly1 it has been clearly demonstrated that it is located in chloroplasts and in the nucleus, respectively, of exactly the same cell [7]. Intriguingly, its nuclear form was observed to have the same molecular weight as the mature plastid form [7]. To investigate whether Whirly1 might get translocated from plastids to the nucleus, a gene cassette encoding an HA-tagged version of *Arabidopsis thaliana* Whirly1 was inserted into the tobacco plastid genome. Immunological analyses with leaves from the transplastomic tobacco plants revealed that the tagged Whirly1 is recruited from chloroplasts to the nucleus where it was shown to affect expression of target genes.

2. Materials and methods

2.1. Plant material

A gene cassette including the cDNA sequence of *Atwhirly1* (TAIR ID: AT1G14410) lacking the plastid targeting peptide was inserted into pBluescript-SK (Supplementary Fig. S1A; see Supplementary Table S1A for sequence of primers). Cloning of the construct and transformation of tobacco, cv. Petite Havana, were carried out as described [10–12].

Tobacco seeds were grown on selective half-strength MS medium (Duchefa, Haarlem, The Netherlands) containing 3% (w/v) sucrose and spectinomycin (500 mg/l). Plants were cultivated under controlled conditions at 22 °C with a photoperiod of 16 h light

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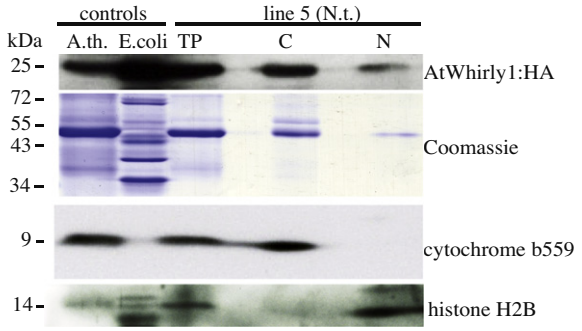

Fig. 1. Immunological detection of HA-tagged AtWhirly1 in chloroplasts and nuclei. Chloroplasts and nuclei fractions were isolated from leaves of transplastomic tobacco line 5. As controls protein extracts from an Arabidopsis line overexpressing an AtWhirly1:HA construct (*A. th.*) and *E. coli* cells expressing the cassette used for plastid transformation (*E. coli*) were used. Loading of the gel was shown by Coomassie staining. Purity of the chloroplast fraction was shown by immunodetection of cytochrome b_{559}. Purity of the nuclear fraction was shown by immunodetection of histone H2B. TP: total protein; C: chloroplasts; N: nuclei.

($100\ \mu E \times m^{-2} \times s^{-1}$). After three weeks of growth seedlings were transplanted to soil and further cultivated in a glass house under natural light conditions.

2.2. Protein extraction and immunological gel blot analysis

Details are given in the Supplementary material and methods.

2.3. Fixation and embedding for microscopy

Details are given in the Supplementary material and methods.

2.4. Immunofluorescence and immunogold labelling

Details are given in the Supplementary material and methods.

2.5. Quantitative RT-PCR analyses

Details are given in the Supplementary material and methods.

3. Results

3.1. Accumulation of plastid-encoded Whirly1 in leaves

To allow to pursue the translocation of plastid Whirly1 to the nucleus a construct encoding an HA-tagged version of AtWhirly1 lacking the transit peptide was inserted into the plastid genome of tobacco according to described methods [10–12]. For selection of transplastomic plants the cassette included the *aadA* gene conferring resistance towards spectinomycin (Supplementary Fig. S1A). In order to ensure insertion into an appropriate position of the inverted repeat by homologous recombination, the cassette was flanked by fragments encoding the 3′ region of the *rps*12 gene and *orf*131, respectively (Supplementary Fig. S1A). Genomic DNA from five independent transplastomic lines was digested with *Eco*RI and analysed by DNA blot analysis using a probe specific for *rps12* (Supplementary Fig. S1B). A 1038 bp fragment obtained by digestion with *Eco*RI indicated integration of the transgene cassette whereas a fragment of 3136 bp was derived from the untransformed plastid genome. Transplastomic lines 1, 2 and 3 seemed to be homoplastomic, whereas line 5 was heteroplastomic (Supplementary Fig. S1B).

Quantitative PCR with DNA from line 5 and primers specific for the *AtWhirly1* gene as well as for selected plastidic (*23SrDNA, psbD*) and nuclear genes (*actin*, *EF-1α*) showed that more than 50% of the plastid genome copies contained the transgene (Supplementary Fig. S1C). Protein extracts from leaves of the five lines were tested for accumulation of the AtWhirly1:HA fusion protein by immunoblot analysis using an antibody specific for the HA-tag. Lines 1, 2, 3 and 5 previously shown to have the correct insertion (Supplementary Fig. S1B) accumulated AtWhirly1:HA having a molecular weight of 25 kDa (Supplementary Fig. S1D). Line 5 was the only line producing seeds and hence was chosen for further analyses.

3.2. Detection of plastid-encoded AtWhirly1 in nuclei

Whirly1 has previously been shown to have the same size in chloroplasts and the nucleus indicating a possible retrograde transfer of the processed protein from chloroplasts to the nucleus [7]. In order to test this hypothesis, chloroplasts and nuclei from leaves of transplastomic tobacco line 5 were prepared. Immunoblot analysis with an antibody specific for the HA-tag revealed that AtWhirly1:-HA synthesised in plastids is contained both in the chloroplast fraction as well as in the nuclear fraction (Fig. 1). As controls a protein extract from rosette leaves of Arabidopsis plants overexpressing the processed form of the HA-tagged AtWhirly1 and a protein extract from *Escherichia coli* cells expressing the gene cassette used for transformation of tobacco were used (Fig. 1). To demonstrate purity of the fractions analogous blots were incubated with antibodies directed against cytochrome b_{559} and histone H2B, respectively. These analyses showed that the plastid fraction does not contain detectable levels of nuclear proteins and the nuclei fraction *vice versa* does not contain detectable levels of plastid proteins (Fig. 1).

To further confirm the dual localisation of the plastid-encoded AtWhirly1:HA in chloroplasts and nucleus, we investigated its subcellular localisation by immunofluorescence using an antibody directed against the HA-tag. Semithin sections were prepared from specimens excised between the major veins of mature leaves of transplastomic tobacco line 5. For detection of AtWhirly1:HA by confocal laser scanning microscopy we used secondary antibodies coupled to Alexa Fluor 568. Immunofluorescence was found to be associated with structures in the cytoplasm and nuclei (Fig. 2A). Nuclei were identified by counterstaining with the DNA-specific fluorescent dye TO-PRO-1 iodide (Fig. 2B). The overlay of fluorescence and phase contrast showed that fluorescence from immune complexes was associated with chloroplasts and nuclei (Fig. 2C). In order to rule out any false signals due to autofluorescence, labelling was additionally performed with gold-coupled secondary antibodies. For visualisation by light microscopy, gold particles were enlarged by silver enhancement and detected by confocal reflection microscopy. By these analyses gold particles were clearly detected in nuclei and chloroplasts (Fig. 2D and E). Taken together these results confirm the presence of HA-tagged AtWhirly1 in both chloroplasts and the nucleus of each cell. Immunogold labelling of ultrathin sections from the same specimen confirmed the presence of AtWhirly1:HA in the nucleus where it seems to associate with the dense chromatin (Fig. 2F). This distribution resembled the distribution of gold particles previously obtained with the antibody directed against the endogenous Whirly1 protein of barley [7].

3.3. Alteration of pathogen response gene expression

Previously, the nuclear form of Whirly1 has been predicted to be a *trans*-acting factor of pathogen response genes (*PR* genes) [1,13]. Quantitative real-time PCR with RNA derived from leaves of wild-type and transplastomic line 5 revealed that relative transcript levels of *PR1* and *PR2* genes were enhanced by factors of more than 700 and 70, respectively, in leaves of transplastomic tobacco line 5 (Fig. 3). In comparison, expression of a nuclear control gene encoding elongation factor 1α (*EF-1α*) was enhanced in the transplastomic line by a factor of 1.5 only (Fig. 3).