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An Alternative Nested Reading Frame May Participate in the Stress-Dependent Expression of a Plant Gene

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Although plants as sessile organisms are affected by a variety of stressors in the field, the stress factors for the above-ground and underground parts of the plant and their gene expression profiles are not the same. Here, we investigated NbKPILP, a gene encoding a new member of the ubiquitous, pathogenesis-related Kunitz peptidase inhibitor (KPI)-like protein family, that we discovered in the genome of Nicotiana benthamiana and other representatives of the Solanaceae family. The NbKPILP gene encodes a protein that has all the structural elements characteristic of KPI but in contrast to the proven A. thaliana KPI (AtKPI), it does not inhibit serine peptidases. Unlike roots, NbKPILP mRNA and its corresponding protein were not detected in intact leaves, but abiotic and biotic stressors drastically affected NbKPILP mRNA accumulation. In search of the causes of suppressed NbKPILP mRNA accumulation in leaves, we found that the NbKPILP gene is “matryoshka,” containing an alternative nested reading frame (ANRF) encoding a 53-amino acid (aa) polypeptide (53aa-ANRF) which has an amphipathic helix (AH).

We confirmed ANRF expression experimentally. A vector containing a GFP-encoding sequence was inserted into the NbKPILP gene in frame with 53aa-ANRF, resulting in a 53aa-GFP fused protein that localized in the membrane fraction of cells. Using the 5′-RACE approach, we have shown that the expression of ANRF was not explained by the existence of a cryptic promoter within the NbKPILP gene but was controlled by the maternal NbKPILP mRNA. We found that insertion of mutations destroying the 53aa-ANRF AH resulted in more than a two-fold increase of the NbKPILP mRNA accumulation. In leaves, we found that the NbKPILP gene but was controlled by the maternal NbKPILP mRNA. We found that insertion of mutations destroying the 53aa-ANRF AH resulted in more than a two-fold increase of the NbKPILP mRNA level.

The NbKPILP gene represents the first example of ANRF functioning as a repressor of a maternal gene in an intact plant. We proposed a model where the stress influencing the translation initiation promotes the accumulation of NbKPILP and its mRNA in leaves.

Keywords: Kunitz peptidase inhibitor, matryoshka gene, alternative nested reading frame, abiotic and biotic stress, virus, bacteria, gene, expression, resistance
INTRODUCTION

Plants in natural field conditions are constantly exposed to a variety of abiotic and biotic environmental factors (Wang et al., 1996; Chaves and Oliveira, 2004; Nakashima and Yamaguchi-Shinozaki, 2006; Hirel et al., 2007; Bailey-Serres and Voesenek, 2008; Atkinson and Urwin, 2012; Suzuki et al., 2014; Ramegowda and Senthil-Kumar, 2015).

A set of stress factors and, accordingly, gene expression profiles for the underground and above-ground parts of plants differ (Freschet et al., 2015; Islam et al., 2015a,b; Liu et al., 2015; Chmielewska et al., 2016). Wind, rain, and herbivorous insects cause mechanical damages mainly to the above-ground part of the plants (Jaffe and Forbes, 1993; Sampathkumar et al., 2014; Savatin et al., 2014; Lup et al., 2016), which increase opportunities for penetration by bacteria and viruses (Carr et al., 2010; Ryu, 2015). Plant roots suffer primarily from soil bacteria (Alegria Terrazas et al., 2016), root-feeding herbivores (Kergunteuil et al., 2016), and salinity (Hanin et al., 2016). Cold, lack of water or lack of nutrients have a generalized effect on the plant (Suzuki et al., 2014; Ramegowda and Senthil-Kumar, 2015).

The plant, in response to the impact of a stress factor, switches on protective mechanisms, including generalized cellular mobilization and accumulation of mRNAs that direct the synthesis of protective proteins in cells (Dowen et al., 2012; Zavaliiev et al., 2013; Crisp et al., 2016; Yang et al., 2017; Zhang and Sonnewald, 2017). Termination of the stress-factor impact cancels the synthesis of protective protein mRNAs and is followed by their degradation. Such repeated effects create a kind of “memory” (Crisp et al., 2016), which allows plants to shorten the time for a protective response. The reaction of the plant genome to the stress is carried out in close interaction with the plastid and mitochondrial genomes via anterograde and retrograde signaling (Leister et al., 2017). A main phytohormone, abscisic acid, plays an essential role in modifying the expression levels of stress-responsive genes (Baek et al., 2017; Vishwakarma et al., 2017). Stress-dependent modification of gene expression can be regulated through transcriptional factors (Eulgem and Somssich, 2007; Jang, 2016; Baek et al., 2017; Fang et al., 2017) and post-transcriptional mechanisms including alternative splicing (Shang et al., 2017) and mRNA nucleocytoplasmic trafficking via the nuclear pores (Yang et al., 2017). The translation of mRNA is also a mechanism for responding to stress, especially the stages involving the 5′ terminal oligopyrimidine tract and translation initiation factors eIF4E and eIF2a (Sesma et al., 2017).

It is known that the same genes related to defense provide a different level of mRNA accumulation in leaves and roots after challenged with abiotic factors (De Nardi et al., 2006; Bazakos et al., 2015; Corso et al., 2015; Freschet et al., 2015; Liu et al., 2015; Pucholt et al., 2015; Chmielewska et al., 2016; Fan et al., 2016; Goralski et al., 2016; Gurung et al., 2016; Miao et al., 2016; Nguyen et al., 2016; Kobayashi et al., 2017). Similar leaf-root differences after viral (Sohal et al., 1999; Germundsson et al., 2002; Andika et al., 2016), fungal (Galiana et al., 1997; Okubara and Paulitz, 2005), bacterial (Zdor and Anderson, 1992; de A Gerhardt et al., 1997; Gruau et al., 2015), and insect attack (Hermsemeier et al., 2001) were noted for various genes such as pathogenesis-related (PR) genes (Fu and Dong, 2013; Parmar et al., 2017) and genes encoding peptidase inhibitors (PIs) (Hörger and van der Hoorn, 2013; Jashni et al., 2015; Gross-Holz and van der Hoorn, 2016; Misas-Villamil et al., 2016). PIs are classified into superfamilies which are subcategorized further into specific families (Rawlings et al., 2014, 2016). Among them is the Kunitz peptidase inhibitor (KPI) family belonging to the serine PI group (https://www.ebi.ac.uk/merops), which includes proteins of ~20 kD with one or two disulfide bonds and a single reactive site (Oliva et al., 2010, 2011; Santamaria et al., 2014; Guo et al., 2015). Functional analysis of the poplar KPI family revealed biochemical diversity and multiplicity in defense reactions (Major and Constabel, 2008). Moreover, a study of white clover has shown that some members of the KPI family are expressed only in roots and not in leaves or other parts of plants (Islam et al., 2015a,b).

Until recently, stress response studies and leaf-root differences in mRNA accumulation studies focused primarily on the mechanisms of transcriptional promoter involvement in gene regulation, activation of transcription factors, and chromatin cytosine methylation in the promoter region of certain genes to prevent the access of transcriptional activators (Komarnytsky and Borisjuk, 2003; Jones and Dangl, 2006; Ryu, 2015; Espinas et al., 2016; Neto et al., 2016; Jiang et al., 2017; Marand et al., 2017). Explanations of the differences in the expression of a gene in leaves and roots are usually based on mechanisms involving transcription factors (Kobayashi et al., 2017), hormones (Fujita et al., 2006), volatile organic compounds (Duran-Flores and Heil, 2016), and mobile RNA transcripts (Saplaura and Kragler, 2016).

Now it has become clear that the leaf-root differences in gene expression and their reactions to stress cannot be explained by only the regulation of gene transcription. So far, no studies have been conducted on alternative nested reading frames (ANRF) in mRNA and their role in mRNA stability to explain the pattern of certain gene expression levels in leaf and root tissues. Although until recently there was an established opinion that one RNA transcript encodes a single protein, the latest data from proteogenomics pointed to the existence of an exception to this rule, which, in many respects, changes the customary meaning of the term “gene” (Kochetov et al., 2013; Liu and Qian, 2014; Mouilleron et al., 2016). The coding potential of such ANRF was not taken into account in most cases, primarily due to the lack of information on the possibility of their translation to polypeptides in a cell (Andrews and Rothnagel, 2014). Recent discoveries of the cell polypeptides encoded by ANRF created the concept of a gene system which could be described as a “matryoshka” (Ribrioux et al., 2008). Improvement of research techniques led to the detection of double-coding transcripts in mammals, such as the TRP-1 gene transcript encoding the TYRP1 tumor antigen (Wang et al., 1996), the caspase 1 (CASP1 or ICE) transcript (Ronsin et al., 1999), the prion protein gene PRNP (Vanderperre et al., 2011, 2013), and the gene encoding Ataxin-1 (ATXN1) (Bergeron et al., 2013).

In principle, besides the ANRF 5′-leader sequence region designated as upstream open reading frames (uORFs), plant
mRNAs contain a plurality of ANRF in the main open reading frame (Hayden and Jorgensen, 2007; Tran et al., 2008; Vaughn et al., 2012). The role of uORF expression in plant stress response was confirmed by ribosomal profiling in normal and stress conditions (Juntawong et al., 2014; Tanaka et al., 2016; Bailey-Serres and Ma, 2017; Schepetilnikov and Ryabova, 2017; Sesma et al., 2017; Xu et al., 2017a,b). Expression of ANRF located in the coding portion of the main gene has only been demonstrated for the maize gene Zm908 (Dong et al., 2013; Hanada et al., 2013).

Here, we identified and described the properties of a novel Nicotiana benthamiana gene encoding a KPI-like protein (NbKPILP). Unlike roots, NbKPILP mRNA and its corresponding protein were not detected in intact leaves, but prolonged darkness and bacterial or viral infection stimulated its mRNA accumulation. We found that the NbKPILP gene is common for Solanaceae plants and belongs to a matryoshka gene family containing an ANRF that encodes a 53-amino acid (aa) polypeptide (53aa-ANRF) which contains an amphipathic helix (AH). Our experimental approaches allowed identification of the 53aa-ANRF that affected the level of NbKPILP mRNA accumulation in intact leaves. The existence of a cryptic promoter within the NbKPILP gene was excluded using the 5'-RACE approach. We found that insertion of mutations destroying the 53aa-ANRF AH resulted in an increase of NbKPILP mRNA accumulation. The NbKPILP gene is the first example of an ANRF influencing maternal mRNA accumulation in leaves.

MATERIALS AND METHODS

Plant Growth Conditions

Nicotiana benthamiana plants were grown in soil in a controlled environment under a 16/8 h day/night cycle.

Plasmid and Vectors

To create 35S-NbKPILP construct NbKPILP-encoding sequence was obtained by PCR using “NbKPILP(KpnI)d” and “NbKPILP(SalI)r” primers and N. benthamiana total cDNA as a template. PCR product was subsequently digested with KpnI and SalI and inserted into pCambia1300-based binary vector containing Cauliflower mosaic virus (CaMV) 35S promoter and 35S terminator of transcription (pCambia-35S) via KpnI/SalI sites. To create 35S-AtKPI construct AtKPI-encoding sequence was obtained by PCR using “AtKPI(SacI)d” and “AtKPI(PstI)r” primers and total A. thaliana cDNA as a template. PCR product was digested with SacI and PstI and inserted into pCambia-35S SacI/PstI sites.

To obtain the (SS)-NbKPILP-6xHis plasmid,SacI, and HindIII sites were introduced at the 5′- and the 3′-ends of (SS)-NbKPILP-encoding sequence, respectively, through PCR with primers “ss-NbKPILP(SacI)d” and “NbKPILP(HindIII)r.” The (SS)-NbKPILP fragment flanked with SacI and HindIII was cloned into pQE30 (QIAGEN, Holland) plasmid digested with SacI and HindIII, to generate the (SS)-NbKPILP-6xHis construct. To obtain the (SS)-AtKPI-6xHis construct (SS)-AtKPI sequence was amplified using the primer pair “AtKPI(BamHI)d” and “AtKPI(HindIII)r.” PCR product was subsequently digested with BamHI and HindIII endonucleases. That fragment was cloned into pQE30 (QIAGEN, Holland) plasmid digested with BamHI and HindIII. The 35S-NbKPILP(53aa-GFP) and 35S-(SS-)NbKPILP(53aa-GFP) constructs the fragments, containing 1–565 or 70–565 nt of NbKPILP ORF, respectively, were amplified using the following pairs of primers: “NbKPILP(KpnI)d”/“53aa_end(BamHI)r” or “ss-NbKPILP(SacI)d”/“53aa_end(BamHI)r.” The PCR products were digested with KpnI/BamHI and together with the fragment containing GFP flanked with BamHI and PstI were inserted into digested with GFP with KpnI and PstI pCambia-35S resulting in 35S-NbKPILP(53aa-GFP) and 35S-(SS-)NbKPILP(53aa-GFP) constructs, respectively.

The 35S-NbKPILP(AGC) construct was created in several steps. First, site-directed mutagenesis using “NbKPILP(KpnI)d”/“53aa(AGC)r” and “53aa(AGC)d”/“NbKPILP(SalI)r” pairs of primers and 35S-NbKPILP plasmid as a template was performed. Second, the final PCR product, containing NbKPILP(AGC) sequence was obtained using overlap PCR approach with “NbKPILP(KpnI)d” and “NbKPILP(SalI)r” primer of primers. The product of overlap PCR was digested with KpnI and SalI and inserted into pCambia-35S via KpnI/SalI sites.

The crTMV-53aa and crTMV-53aa_mut vectors were created in several steps. First, a PCR fragment containing 53aa-ANRF-encoding sequence (407–568 nt of NbKPILP ORF) was obtained using “53aa(NcoI)d” and “53aa(XhoI)r” pair of primers. For substituted variant (53aa_mut) the PCR fragment was obtained using “53aa(NcoI)d”/“53aa(AHmut)r” and “53aa(AHmut)d”/“53aa(XhoI)r” pair of primers for site directed mutagenesis and “53aa(NcoI)d”/“53aa(XhoI)r” pair of primers for overlap PCR. The resulting PCR products, encoding 53aa-ANRF or 53aa_mut, were digested with NcoI and XhoI and inserted into plasmid pCMV-1C-2 (Komarova et al., 2011), containing 3′-region of crTMV movement protein (MP) gene, 3′-UTR of crTMV and nos transcription terminator. The resulting constructs were digested with EcoRI/Xhol and a fragment containing part of MP, 53aa-ANRF (or 53aa_mut), crTMV 3′-UTR and nos terminator was inserted into TMV-HC vector (Komarova et al., 2011) via EcoRI/Xhol sites resulting in crTMV-53aa or crTMV-53aa_mut construct, respectively.

To obtain the plasmid encoding NbKPILP(53aa) fused to the DYKDDDKDDKDYKDDDKDDDK (3xFLAG) sequence (Ueda et al., 2011), a fragment containing full NbKPILP sequence without stop codon was amplified using “NbKPILP(KpnI)d” and “NbKPILP(XbaI)r” primers and subsequently digested with KpnI and XbaI. The 3xFLAG encoding sequence was generated annealing the primers “3xFlag(XbaI)d” and “3xFlag(SalI)r,” resulting in a fragment with overhangs corresponding to XbaI and SalI “sticky” ends. These fragments were inserted into pCambia-35S via KpnI/SalI sites to generate the 35S-NbKPILP(53aa):3xFLAG plasmid. For 35S-NbKPILP(53aa_mut):3xFLAG construct, fragment contained NbKPILP(53aa_mut) was obtained by site-directed mutagenesis using primer pairs “NbKPILP(KpnI)d”/“53aa(AHmut)r” and “53aa(AHmut)d”/“NbKPILP(XbaI)r” and 35S-NbKPILP as a template. Then these PCR fragments were used in overlap PCR with “NbKPILP(KpnI)d”/“NbKPILP(XbaI)r” primers. The
resulting product of overlap PCR was digested with KpnI and XbaI and inserted into pCambia-35S together with 3xFLAG-encoding fragment flanked with XbaI and SalI resulting in 35S-NbKPILP(53aa_mut):3xFLAG.

A full list of the oligonucleotides used for cloning is presented in Table S1.

**Transcription Start Site Determination Using a Step-Out Rapid Amplification of cDNA 5′-End (5′-Race) Approach**

The 5′-RACE of NbKPILP cDNA was performed using the Mint RACE cDNA amplification set (Evrogen, Russia) according to manufacturer's instructions. The following gene-specific primers were used: "pr1;" "pr2" (Table S1).

**Agroinjection Experiments**

The Agrobacterium tumefaciens strain GV3101 was transformed with individual constructs and grown at in LB medium supplemented with 50 mg/l rifampicin, 25 mg/l gentamycin, and 50 mg/l kanamycin. The Agrobacterium from an overnight culture were resuspended in 10 mM MES buffer (pH 5.5) supplemented with 10 mM MgSO4 and adjusted to a final OD600 of 0.1. Agroinjection was performed using nearly fully expanded N. benthamiana leaves attached to the intact plant. A bacterial suspension was infiltrated into the leaf tissue using a 2-ml syringe, after which the plants were grown under greenhouse conditions at and a 16/8 h light/dark photoperiod unless otherwise specified.

**N. tabacum Inoculation with TMV**

Lower leaves of N. tabacum cv. Samsun plants were mechanically inoculated with TMV virions (100 mg/ml) in 50 mM sodium phosphate buffer, pH 7.0, in the presence of Celite, as described previously (Dorokhov et al., 1981).

**GFP Visualization**

GFP fluorescence in the inoculated leaves was monitored by illumination with a handheld UV source, λ = 366 nm. To visualize 53aa-GFP subcellular localization, imaging was performed 72 h after agroinfiltration with 35S-NbKPILP(53aa-GFP) in the presence of a vector encoding silencing suppressor P19 from Tomato bushy stunt virus using an LSM510 confocal laser scanning microscope (Zeiss, Germany). Excitation wavelength was 488 nm and detection window was 493–550 nm.

**Western-Blot Analysis**

For Western blot analysis, the proteins from agroinjected leaves were divided into S17, P17, P1, and CW crude fractions according to Deom et al. (1987) with modifications. Briefly, frozen plant material was ground to a powder in liquid nitrogen followed by addition of three volumes of ice-cold-modified GB buffer (100 mM Tris, pH 8.0, 0.4 M sucrose, 10 mM KCl, 5 mM MgCl2, 10 mM β-mercaptoethanol). The obtained slurry was filtered through a double-layered Miracloth (Millipore/Merck). Then the material retained on the filter was collected and washed (30–60 min incubation followed by centrifugation at 1,000 × g) 5–8 times with GB-buffer supplemented with 0.1% Triton X-100 (the final wash was performed without Triton X-100) to obtain the CW-enriched fraction. The filtrate was centrifuged at 1,000 × g for 10 min to obtain the P1 (pellet enriched with nuclei) fraction. Supernatant was further divided into S17 (supernatant enriched with soluble proteins) and P17 (pellet enriched with membrane non-soluble proteins) fractions after centrifugation at 17,000 × g for 30 min. Pellets from CW, P1 and P17 fractions were resuspended in one volume of 1xPBS.

Total protein was extracted from plant tissues using TriReagent (MRC) according to the manufacturer's instructions. Aliquots from all fractions were analyzed through SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes (GE Healthcare). For GFP or NbKPILP detection, the membranes were probed with corresponding antibodies: goat anti-GFP antibodies conjugated with horseradish peroxidase (Rockland Immunochemicals) or mouse polyclonal antibodies against recombinant NbKPILP6His. Anti-mouse antibodies conjugated with horseradish peroxidase (Rockland Immunochemicals) were used as secondary antibodies. The bands were visualized using the chemiluminescence ECL kit (GE Healthcare).

**Q-PCR Analysis of Transcript Concentrations**

Total RNA was extracted from plant tissues using TriReagent (MRC) according to the manufacturer's instructions. The RNA concentration was determined using a Nanodrop ND-1000 spectrophotometer (Isogen Life Sciences). All RNA samples had a 260:280 absorbance ratio between 1.9 and 2.1. The synthesis of the first strand, followed by real-time qPCR, was performed as described in Dorokhov et al. (2012). Briefly, 0.1 mg of random hexamers and 0.1 mg of oligo-dT primer were added to 2 mg of total RNA to obtain cDNA through reverse transcription using Superscript II reverse-transcriptase (Invitrogen), according to the manufacturer's protocol. Real-time quantitative PCR was carried out using the iCycler iQ real-time PCR detection system (Bio-Rad). Target genes were detected using sequence-specific primers (Table S2) and Eva Green master mix (Syntol) according to the manufacturer's instructions. Each sample was run in triplicate, and a non-template control was added to each run. A minimum of five biological replicates were performed.

**Statistics**

Student's t-test was performed using Excel (Microsoft, Redmond, WA). P < 0.05 were considered significant.

**RESULTS**

**Identification and Analysis of the N. benthamiana Gene Encoding KPI-Like Protein (NbKPILP)**

In a study of the transcriptome of N. benthamiana leaves infected with Tobacco mosaic virus (TMV), we detected an increased accumulation of mRNA (EMBL ID FN687760) encoding KPI-like protein (NbKPILP) (NCBI accession number D41HB9), which according to the MEROPS classification (http://merops.sanger.ac.uk; Rawlings et al., 2014, 2016) belongs to the 13
family (clan IC), of which the soybean Kunitz trypsin inhibitor
(Glycine max) is a typical member. To determine if there are any
introns present within the NbKPILP ORF, the N. benthamiana
genomic DNA and primers designed for the cDNA amplification
were used to amplify the corresponding fragment. The amplified
fragment length was 606-bp for both genomic DNA and
cDNA. The genomic fragment sequence perfectly matched the
cDNA-derived fragment (data not shown), which indicated the
absence of an intron in the ORF-encoding part of chromosomal
NbKPILP gene. Thus, like other plant KPIs (Jofuku and Goldberg,
1989; Ishikawa et al., 1994; Saarikoski et al., 1996; Gruden
et al., 1997; Ashida et al., 2000; Tian et al., 2007; Huang
et al., 2010; Bhattacharjee et al., 2014; Bunyatang et al., 2016),
the NbKPILP gene does not contain introns, as shown by the
chromosomal DNA sequence that we identified (data not shown)
and a sequence search in the Sol Genomics Network
(http://solgenomics.net/) and the N. benthamiana Genome and
Transcriptome database (http://benthgenome.qut.edu.au/).
The predicted amino acid sequence has high homology to KPI-LP of
other Solanaceae species, including not only the earlier described
N. glutinosa biotic cell death-associated protein (NgCDM1;
Suh et al., 2003) but also KPI-LPs sequences from tobacco,
potato, tomato, and pepper (Figure 1). NbKPILP contains a
predicted 24-aa signal sequence and has homology to members
of the I3 family such as soybean KTI which possesses inhibitor
activity against serine peptidase and is encoded by the KTI3
gene (GmKTI3) (29.17% identity; Jofuku and Goldberg, 1989),
N. tabacum KPI1 (Huang et al., 2010), and the A. thaliana
KPI (EMBL ID Q8RXD5) (33.3% identity; Li et al., 2008).

Figure 1 also shows alignment of NbKPILP with other sequences
from the N. benthamiana genome: genes encoding miracidin-
like protein 1, NbMLP1 (39.47% identity), NbMLP2 (35.75%
identity), and NbMLP3, which is completely identical to
NbKPILP (100.00% identity; Goodwin et al., 2012). Using the
COBALT multiple alignment tool (https://www.ncbi.nlm.nih.
gov/tools/colalt/cobalt.cgi), a phylogenetic tree including KPIs
and KPI-LPs with highest sequence identity to NbKPILP, was
constructed (Figure S1). Inspection of the phylogenetic tree
revealed that NbKPILP belongs to the same branch as other
KPI-LPs from Solanaceae plants.

The NbKPILP likely undergoes glycosylation similar to
Synsepalum dulcificum miracidin (MIRA) (Takahashi et al., 1990)
which shows the presence of N-linked glycans at the Asn-71
and Asn-215 positions (the numbering includes signal sequences
(Figure 1). NbKPILP has putative N-glycosylated sites in Asn-60,
Asn-86, and Asn-136 (Figure 1) according to a prediction by
the NetNGlyc programme (http://www.cbs.dtu.dk/services/
NetNGlyc/).

The NbKPILP has main sequence signatures of the KPI family
(Major and Constabel, 2008; Huang et al., 2010; Guo et al., 2015)
such as (i) the Kunitz motif, (ii) four cysteine residues arranged
into two intra-chain disulfide bridges, and (iii) the reactive loop.
However, there are significant differences in comparison with
GmKPI3, AtKPI, or NbMLP1, for example (Figure 1). NbKPILP
contains a lysine residue instead of glycine in the Kunitz motif
and substitutions in the P1 reactive site that determines substrate
specificity (Ser instead of Arg or Lys and Ala instead of Ile/Val;
Goodwin et al., 2012; Guo et al., 2015). The reactive loop of
NbKPILP, according to a prediction by PeptideCutter (http://
www.expasy.org/tools/peptidecutter/), does not contain a P1
reactive site residue that interacts with trypsin, such as Arg63-
Ile64 in the Kunitz-type soybean trypsin inhibitor (De Meester
et al., 1998). Thus, NbKPILP is unlikely to have trypsin inhibitory
activity. To test this prediction, we obtained a recombinant E.
coli strain producing NbKPILP containing 6xHis tag and lacking the
signal sequence [(SS-)NbKPILP-6xHis]]. (SS-)AtKPI-6xHis
was used as a positive control with experimentally proven KPI
activity in vitro (Li et al., 2008). Two substrates were used to
evaluate the inhibitory activity of proteins: (i) p-nitroaniline,
a synthetic substrate of trypsin which is characterized by high
specificity of trypsin/substrate interaction and (ii) azocasein, a
natural substrate of trypsin. (SS-)AtKPI-6xHis demonstrated a
90% decrease in protease activity with the synthetic substrate,
and a 55% reduction with the natural substrate whereas (SS-)
NbKPILP-6xHis did not show any trypsin inhibition on either
substrate in vitro (data not shown).

**NbKPILP mRNA Accumulation in N. benthamiana Leaves Is Increased after Incubation in Darkness and Foreign Protein Overproduction**

Biologically, the expression of KPI and KPI-LP in an intact
plant varies widely in roots and leaves (Huang et al., 2010;
Goodwin et al., 2012). We examined adult plants of N.
benthamiana (Figure 2A) and did not identify NbKPILP in
leaves using Western-blott analysis (Figure 2B). Conversely,
the protein analysis of the roots detected a set of protein bands,
among which there was a major double band corresponding
to proteins of 30 and 29 kDa, which significantly exceeds the
predicted weight of a mature NbKPILP (19 kDa). Subcellular root
fractionation showed the presence of NbKPILP mainly in the
membrane fraction (data not shown), which is consistent with
the presence of a signal sequence in the protein (Figure 1). It
can be assumed that the significant differences in the content
of NbKPILP in leaves and roots may occur due to differences
in the level of accumulation of NbKPILP mRNA. We explored
the NbKPILP mRNA levels in roots and mature leaves of healthy
intact plants and showed that NbKPILP mRNA content in
leaves was negligible, while in the roots it was almost three
orders higher (Figure 1). NbKPILP mRNA content in
leaves was low and independent of plant age, while the
NbKPILP mRNA content in roots increased with plant age and was
highest at the flowering stage (Figure S4).

Thus, the intact N. benthamiana roots and leaves are radically
different in NbKPILP content due to differences in the level of
NbKPILP mRNA accumulation.
Fig. 1 Amino acid sequence alignment of NbKPILP with predicted aminoc acid sequences of other homologous proteins. The alignment was performed using the CLUSTAL programme (http://www.ebi.ac.uk/Tools/msa/clustalo). KP-like proteins, CaKPILP (Capsicum annuum KPILP, sequence ID Ntab-BX_AWOK-SS1956), N. benthamiana miraculin-like proteins (NbMLP1, 2, and 3) (Goodwin et al., 2012) and NtKPILP (N. tabacum KPILP, sequence ID Ntab-BX_AWOK-SS1956) were retrieved from the Sol Genomics Network (http://solgenomics.net/). SlKPILP, Solanum lycopersicum KPI-like protein (accession number K4BJT7) and NgKPILP, N. glutinosa biotic cell death-associated protein (accession number Q850R9) were retrieved from the NCBI database. GmKPI3, Glycine max KPI encoded by the KTI3 gene (NCBI accession number P01070) (De Meester et al., 1998), N. tabacum KPI1 (NCBI accession number B8Y888) (Huang et al., 2010), AtKPI, Arabidopsis (Continued)
We assumed that like other KPIs (Huang et al., 2010) and KPILP (Suh et al., 2003; Goodwin et al., 2012; Islam et al., 2015a), NbKPILP mRNA accumulation is stimulated by the action of abiotic and biotic factors. Therefore, we investigated the effect of abiotic factors such as prolonged darkness on the accumulation of NbKPILP mRNA in leaves. For this, we incubated the plants in darkness for different periods and analyzed NbKPILP mRNA content in leaves. Figure 3 shows that after 48 h of darkness NbKPILP mRNA content of the leaves significantly increased and continued to increase up to 72 h, reaching almost a 50-fold increase after a 96-h darkness period. However, when the plants were transferred back to a normal light/darkness photoperiod, NbKPILP mRNA content returned to the original level. Therefore, prolonged exposure of the plants to darkness induces NbKPILP mRNA accumulation in leaves.

We expected that biotic factors such as TMV infection, which is associated with impaired functioning of the chloroplast and the appearance of mosaic symptoms, should lead to the stimulation of NbKPILP mRNA accumulation. To test this hypothesis, we used tobacco (Nicotiana tabacum L. cv. Samsun) systemically infected with TMV, whose upper leaves showed mosaic symptoms, i.e., alternation of dark green spots (islands, as described by Atkinson and Matthews, 1970) characterized with a low content of TMV and light, yellow-green leaf areas enriched with TMV particles (Atkinson and Matthews, 1970; Moore et al., 2001; Figure 4A). Our analysis of both regions of mosaic leaves revealed a huge amount of TMV coat protein accumulated in yellow-green islands (data not shown) and a significant increase in NbKPILP mRNA content in yellow-green areas compared with dark green islands and especially compared with leaves of the uninfected plants (Figure 4B).

Synthesis of a foreign protein in N. benthamiana leaves may also lead to the stimulation of NbKPILP mRNA accumulation. We chose GFP as a foreign protein, and used two types of crTMV-based vectors (Figure 5A) with different productive capacities for GFP synthesis in plants. The control vector, crTMV-GFP, provided moderate production of GFP (Figure 5B) and its-modified variant, crTMV(i)-GFP, containing multiple introns in the crTMV cDNA sequence (Komarova et al., 2012), mediated excessive GFP production in leaves 4 days after agroinjection (Figure 5B). In parallel with the increase in accumulation of GFP mRNA (Figure 5), NbKPILP mRNA content also increased (Figure 5D).

We concluded that factors of abiotic and biotic stress stimulate NbKPILP mRNA accumulation in the leaves.

### NbKPILP Is a Matryoshka Gene Containing a Translatable, Alternative Nested Reading Frame Encoding a 53-aa Polypeptide (53aa-ANRF)

Biologically, the amount of NbKPILP in leaves can be determined by both the transcriptional activity of the gene and the stability of its mRNA in the cytoplasm. Recently, the role of upstream open reading frames (uORFs) in 5′-UTRs in mRNA translation and stability was shown (Tanaka et al., 2016; Bailey-Serres and Ma, 2017; Xu et al., 2017b). To identify putative uORFs, we identified the transcription start site and the sequence of the
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FIGURE 3 | Darkness stimulates the accumulation of NbKPILP mRNA in the leaves. Plants were kept in darkness for 24, 48, 72, or 96 h, and the leaf samples were taken for analysis of NbKPILP mRNA by qRT-PCR. The data shown represent five independent experiments. The difference in NbKPILP mRNA content between control plants and plants after different periods of incubation in darkness (24, 48, 72, 96, and 24 h after return to a normal photoperiod) is shown. *** P < 0.001, n.s. = not significant (Student’s t-test).

FIGURE 4 | The yellow-green areas of TMV-infected leaves with mosaic symptoms are characterized by a high content of NbKPILP mRNA. (A) Mosaic symptoms of N. tabacum cv. Samsun leaves systemically infected with TMV. (B) Relative amounts of NbKPILP mRNA in dark-green and yellow-green areas of leaves with mosaic symptoms quantified by qRT-PCR. The data represents five independent experiments, and the standard error bars are indicated. The statistical significance of the difference in NbKPILP mRNA content between mosaic areas and control non-infected leaves is shown. *** P < 0.001 (Student’s t-test).

NbKPILP mRNA 5’-UTR using the 5’-Rapid Amplification of Complementary Ends (5’-RACE) approach. As NbKPILP mRNA content in the intact leaf is very low (Figure 2), we used two approaches to increase it: (a) incubation of the plants in darkness for 4 days according to the scheme shown in Figure 3 and (b) agroinjection of N. benthamiana leaves with a plasmid directing NbKPILP mRNA synthesis.

To obtain cDNA suitable for the identification of the 5’-end of NbKPILP mRNA, we used the template-switching principle to add an adapter sequence to the 5’-end of the cDNA (Matz et al., 1999). Then, we performed PCR with sequence-specific reverse primers, pr1 or pr2, complementary to the NbKPILP region of mRNA, and a forward primer corresponding to the adapter sequence (Figure 6A). We obtained only one PCR product for each reverse primer (pr1 or pr2; Figure 6B). Sequencing of these products allowed us to identify the transcription start site and 5’-UTR (Figure 6). The mRNA transcription start site is located 23 nts upstream of NbKPILP ORF and contains a uORF with the sequence ATGCATTAA (Figure 7A). Although it is known that such start-stop codons in the 5’-UTR are able to influence the stability of mRNA (Tanaka et al., 2016), we are sure that this is not the case, since the uORF is located directly at the 5’-end. In fact, in agroinjection experiments, comparison of constructs with or without this uORF did not reveal differences in the accumulation of NbKPILP mRNA in the leaves (data not shown).
However, looking closer at the mechanism of NbKPILP mRNA translation, we see that the first AUG codon (Figure 7A) is in an unfavorable context and the ribosomal 43S Pre-Initiation Complex (PIC) is likely to skip this codon via the leaky scanning mechanism (Kozak, 1986). For dicots, aaA(A/C)aAUGGCu is the optimal context for the start AUG codon (agAaaAUGG; Figure 7A). For dicots, aAUGGCU is also optimal for translation (agAaaAUGG; Figure 7A). However, looking closer at the mechanism of NbKPILP mRNA translation, we see that the first AUG codon (Figure 7A) is in an unfavorable context and the ribosomal 43S Pre-Initiation Complex (PIC) is likely to skip this codon via the leaky scanning mechanism (Kozak, 1986). For dicots, aaA(A/C)aAUGGCu is the optimal context for the start AUG codon (agAaaAUGG; Figure 7A). For dicots, aAUGGCU is also optimal for translation (agAaaAUGG; Figure 7A).

According to a prediction by the Phyre2 programme, the 53aa-ANRF contains a transmembrane amphipathic helix (Table 1, Figure S5) indicating that the hypothetical 53-aa polypeptide is targeted to the cell membrane. To identify whether the 53-aa polypeptide is synthesized, we used an experimental approach in which we fused the 53aa-ANRF coding frame with the GFP gene, following the general design and context of the NbKPILP gene. This technique would allow us to detect 53aa-GFP using a Western blot assay in different cell fractions. We created two vectors encoding the N-terminal part of NbKPILP up to the end of the 53aa-ANRF fused to GFP with a signal sequence [35S-NbKPILP(53aa-GFP)] and without a signal sequence [35S-(SS)-NbKPILP(53aa-GFP)] (Figure 7B). Then, N. benthamiana leaves were agroinjected with these constructs and GFP synthesis was examined by fluorescence microscopy which revealed a small amount of fluorescent single cells in leaves (Figure S6). To make sure that the cells synthesized GFP fused with 53-aa polypeptide, we performed a Western-blot analysis of subcellular fractions of leaves agroinjected with the abovementioned binary vectors. Figures 7D,E show that in accordance with the predicted amphipathic properties, GFP fused with the 53-aa polypeptide was detected mainly in the cell wall fraction. Its synthesis can be seen in the leaves agroinjected with constructs encoding NbKPILP(53aa-GFP) with (Figure 7D) and without a signal sequence (Figure 7E).

The results of 5’-RACE confirmed that no additional shorter variants of NbKPILP cDNA detected in the cell (Figure 6B),
therefore the expression of ANRF could not be explained by the existence of a cryptic promoter within the NKPILP gene but is likely to be controlled by the maternal NKPILP mRNA.

We concluded that NKPILP mRNA is capable of directing the synthesis of the 53-aa polypeptide in vivo.

The Role of 53aa-ANRF in the Accumulation of NKPILP mRNA in N. benthamiana Leaves

To assess whether ANRF expression affects the level of mRNA accumulation in the leaves, we used two approaches. First, we assumed that the AtKPI gene, which does not contain ANRF according to predictions (Table 1), is probably expressed more actively in N. benthamiana leaves than NKPILP. We compared the level of mRNA accumulation in agroinjected leaves expressing NKPILP or AtKPI from 35S-based constructs (Figure 8A). Figure 8B shows that the level of AtKPI mRNA in agroinjected N. benthamiana leaves is almost two orders higher than NKPILP mRNA.

Then, we hypothesized that the optimal context of the 53aa-ANRF start codon makes it more preferable for scanning PIC, resulting in suppression of recognition and translational initiation from the NKPILP AUG codon. To test this hypothesis, we inserted a nucleotide substitution into the ANRF AUG start codon, resulting in an ACG codon (Figure 9A) and thus lowering the probability of 53aa-ANRF translation. Figure 9B shows that leaf agroinjection with 35S-NKPILP(ACG) resulted in a 10-fold increase in mRNA content in comparison to 35S-NKPILP(ATG). Moreover, the results of a Western-blot analysis of leaf proteins were consistent with the mRNA analysis (Figure 9). Antibodies to NKPILP-6xHis have not revealed NKPILP-specific proteins in intact leaves. Only after leaf agroinjection with 35S-NKPILP did we detect a weak signal of the 30-kDa protein. For the mutant 35S-NKPILP(ACG), however, we observed very intense protein bands, including three major and two minor protein bands, the presence of which is likely due to the manifestation of various predicted NKPILP glycoforms (Figure 1).

We concluded that the accumulation of NKPILP and its mRNA depends on the expression of 53aa-ANRF.

Effect of the 53aa-ANRF Amphipathic Helix (AH) on NKPILP Expression

It can be assumed that the inhibitory effect of ANRF on the accumulation of mRNA in the intact leaf is related to the amphipathic nature of the 53-aa polypeptide (Figure S5). It is known that the synthesis of a protein containing AH leads to endoplasmic reticulum membrane modification and
FIGURE 7 | NbKPILP mRNA contains an ANRF translated into short 53-aa polypeptide (53aa-ANRF) in N. benthamiana leaves. (A) The sequence of the NbKPILP cDNA with the designated potential start codons (bold). Polyurine block sequence is highlighted in bolded red; 53aa-ANRF is italicized and underlined. (B,C) Schematic representation of the 35S-NbKPILP(53aa-GFP) (B) and 35S-(SS-)NbKPILP(53aa-GFP) (C) vectors comprising the following elements: CaMV 35S promoter, NbKPILP with a deleted 24-aa signal sequence (SS-) and 53aa-ANRF fused with GFP. (D,E) Western blot analysis using anti-GFP antibodies of N. benthamiana leaf proteins at 3 days after agroinjection with the 35S-NbKPILP(53aa-GFP) (D) or 35S-(SS-)NbKPILP(53aa-GFP) (E) vectors. Cell homogenate was fractionated into P1, P17, and S17 fractions. G, the total soluble protein from leaves 3 days after agroinjection with 35S-GFP vector. The lower panels show the protein loading control stained with Amido Black. All agroinfiltrations were performed in the presence of a vector encoding silencing suppressor P19 from Tomato bushy stunt virus.

TABLE 1 | KPILP matryoshka genes with embedded ANRFs.

| # | Plant and protein name | Leaf expression | EMBL ID | Identities*, % | Length of the corresponding protein (aa) | ANRF-encoded peptide contains AH** |
|---|------------------------|-----------------|---------|----------------|------------------------------------------|-----------------------------------|
| 1 | Nicotiana benthamiana KPI (NbKPI) | No | FN687760 | 100 | 201 | Yes |
| 2 | Nicotiana glutinosa KPI (NgKPI) | No | AF208022 | 94.5 | 229 | Yes |
| 3 | Solanum tuberosum KPI (StKPI) | NA | XP_006353918 | 85.2 | 198 | Yes |
| 4 | Solanum lycopersicum KPI (SlKPI) | NA | XM_004235444 | 84.8 | 199 | Yes |
| 5 | Trifolium repens KPI4 (TrKPI4) | No | KF022201.1 | 28.0 | 210 | Yes |
| 6 | Hevea brasiliensis KPI (HbKPI) | NA | EM_PL:KM979450 | 36.5 | 196 | Yes |
| 7 | Arabidopsis thaliana KPI (AtKPI) | NA | At1g73260 | 33.3 | 205 | No |

*Compared to NbKPI.

**Predicted by http://www.cbs.dtu.dk/services/TMHMM-2.0/.
to cell death (Hashimoto et al., 2015). To test the potential ability of 53aa-ANRF to cause cellular death, we created two viral vectors based on the crTMV genome (Figure 10A). In the first vector, crTMV-53aa, the coat protein gene was replaced by a sequence encoding 53aa-ANRF. The second vector, crTMV-53aa_mut, encoded a mutant of 53aa-ANRF (53aa-ANRF_mut) containing amino acid substitutions (Figure 10B) that led to the destruction of the AH (Figure S7). Figure 10C shows that the agroinjection of a half-leaf by the crTMV-53aa vector led to necrosis. However, crTMV-53aa_mut, encoding the 53-aa polypeptide without AH, did not induce cell death.

We concluded that 53aa-ANRF is potentially capable of influencing the membrane structure and potentially playing a role in cell death.

In the next step, we investigated the effect of AH-containing 53aa-ANRF membrane protein synthesis on maternal NbKPILP mRNA accumulation in the leaves. To perform agroinjection experiments, we created a 35S-based vector 35S-NbKPILP(53aa):3xFLAG (Figure 11A) which has the natural organization of NbKPILP mRNA and allows one to distinguish

**FIGURE 8** Comparison of NbKPILP-encoding mRNA containing ANRF and AtKPI-encoding mRNA lacking ANRF accumulation in agroinjected N. benthamiana leaves. (A) Schematic representation of the 35S-NbKPILP and 35S-AtKPI constructs. The nucleotide context of the start codon is indicated. SS, signal peptide encoding sequence. (B) Relative amount of NbKPILP and AtKPI mRNA after agroinjection of the corresponding constructs. The level of NbKPILP mRNA before agroinjection was set as 1. The mean values with bars indicating SE are shown. **FIGURE 9** Inactivation of ANRF dramatically increases the accumulation NbKPILP mRNA in the leaves. (A) Schematic representation of a 35S-NbKPILP vector [35S-NbKPILP(ATG)], and 35S-NbKPILP(ACG) with the 53aa-ANRF ATG start codon substituted with ACG. (B) NbKPILP mRNA accumulation in N. benthamiana leaves 48 h after agroinjection with 35S-NbKPILP(AG) and 35S-NbKPILP(ACG), and quantified by qRT-PCR. The values of samples taken just after agroinjection were set as 1. The data represent five independent experiments, and the standard error bars are indicated. The P-values were used to assess the statistical significance of differences in NbKPILP mRNA levels after agroinjection with 35S-NbKPILP(ACG) compared to 35S-NbKPILP(AG). (C) Western blot analysis of total N. benthamiana leaf proteins at 48 h after agroinjection with the 35S-NbKPILP(AG) or 35S-NbKPILP(ACG) vectors using anti-NbKPILP-6xHis antibodies. rNbKPILP, recombinant NbKPILP with 6xHis-tag produced in E. coli. The lower panels show the protein loading control stained with Amido Black.
between the synthesis of endogenous NbKPILP mRNA and synthesis of mRNA directed by the 35S-based expression vector. Of note, the injection of Agrobacterium leads to the accumulation of endogenous NbKPILP mRNA, and, hence, it is necessary to distinguish the experimental mRNA from the endogenous NbKPILP mRNA. For this purpose, we added a 3XFLAG-tag encoding sequence (Ueda et al., 2011) to the 3′-terminal end of NbKPILP to obtain an NbKPILP:3XFLAG fusion protein.

To understand the effect of AH on the accumulation of NbKPILP mRNA, we created a 35S-NbKPILP(53aa_mut)-3XFLAG, that encoded a 53aa-ANRF_mut with amino acid substitutions that cause destruction of AH, but the nucleotide sequence was mutated in a way that preserved the natural amino acid sequence of NbKPILP. To study the relative amount of NbKPILP:3XFLAG mRNA expression, we performed qRT-PCR using FLAG-specific primers (Table S2). Figure 11B shows that removal of the AH from the 53aa-ANRF resulted in more than a two-fold increase in the NbKPILP:3XFLAG mRNA level.

We concluded that the amphipathic nature of the 53-aa polypeptide is responsible for inhibition of NbKPILP mRNA accumulation in leaves of intact plants.

**DISCUSSION**

Plant KPIs are normally represented by multigene families ranging from several members to several tens of members, as in the case of the poplar (Populus trichocarpa), where a wide range of within-family identity was found from low (38%) to high homology (96%) (Major and Constabel, 2008; Philippe et al., 2009). Some members of this family show their activity in organs only under stressful conditions (Rustgi et al., 2017). The upstream regions of the NbMLP1, NbMLP2, and NbMLP3 genes were identified, and promoter elements responsible for defense responses were studied to explain the activation of these genes during stress (Goodwin et al., 2012). Here, our studies of the N. benthamiana genome revealed a matryoshka gene organization and another way of regulating gene activity in which ANRF functions as a repressor of a maternal gene in the plant. The NbKPILP gene is not unique and its expression pattern indicates no detectable mRNA accumulation in leaves of intact plants, which is also characteristic of other KPIs such as NtKPI1 (Huang et al., 2010), NgKPILP (Suh et al., 2003), TrKPI4 (Islam et al., 2015a), and NbMLP1, NbMLP2, and NbMLP3 (Goodwin et al., 2012). Our analysis revealed that the NbKPILP gene belongs to a six-member group, which includes NgKPILP, StKPILP, SIKPILP, TrKPI4, and HbKPI (Table 1, Table S4). All these genes are characterized by (i) the presence of an ANRF whose start codon is in a more favorable context than the start codon of the main ORF, (ii) the presence of an 18- to 25-nucleotide polypurine block ~30 nt upstream of ANRF, and (iii) the 30- to 53-amino acid long membrane protein encoded by the ANRF.

In principle, the matryoshka gene design can manifest itself on two levels. In the first level, the chromosome level,
Interestingly, unlike in plants, animal genes that encode KPI contain introns (He et al., 2003; Chakrabarty et al., 2006; Isaeva et al., 2012; Chen et al., 2013) and with the help of alternative splicing, can control KPI expression (Sandbrink et al., 1994; Itoh et al., 1999; Kirchhofer et al., 2003; Wood et al., 2014). In plants, there is no alternative splicing for KPIs, so the function of ANRF during mRNA translation can be a mechanism involving stress-regulated gene expression. To explain the low content of NbKPILP and its mRNA in intact leaves, we propose mechanisms that allow the synthesis of the 53-aa polypeptide which modifies ER-membranes in cells of intact leaves (Figure 12). In general, eukaryotic translational initiation is a multistep process that includes PIC assembly, its attachment to the mRNA, mRNA circularization, scanning, start codon selection, and 60S subunit joining (Haimov et al., 2015; Sesma et al., 2017). The first probable mechanism of the 53-aa polypeptide synthesis is based on ribosomal leaky scanning (Figure 12A). Usually, translation is initiated at the first 5′-proximal AUG codon in an acceptable context. The extent of “leakiness” depends on the nucleotide context surrounding AUG. The first AUG codon of NbKPILP mRNA is not in the optimal context for dicots (AANAUGGC) (Figure 12A-1; Kim et al., 2014; Gupta et al., 2016), therefore, the formation of the initiating complex is more preferable at the second AUG initiating codon, which is in the favorable context (Figure 12A-2). PIC scanning for over 400 nucleotides is likely to create the risk of ribosome stalling and mRNA degradation (Kawaguchi and Bailey-Serres, 2005; Hsu et al., 2016). Ribosome shunting, which occurs when the ribosome bypasses or shunts parts of the 5′-UTR on its way to the AUG start codon, is unlikely here (Miras et al., 2017; Terenin et al., 2017) since there is a PP block 39 nts upstream of the ANRF AUG codon which can perform the role of an internal ribosomal binding site (Dorokhov et al., 2002; Figure 12A-3). In either case, both mechanisms could lead to the synthesis of the 53-aa polypeptide, whose AH is potentially capable of ER-membrane modification, i.e., polyribosome detachment leading to mRNA degradation (Hashimoto et al., 2015; Figure 12A-4). Under stress conditions, the synthesis of the 53-aa polypeptide and the degradation of NbKPILP mRNA is downregulated, since the initiation of translation is carried out at the first AUG codon, even in the unfavorable context (Figure 12B). As our experiments showed, stress factors affected the functioning of chloroplasts. It can be assumed that the change in the mode of translation of NbKPILP mRNA under stress is associated with the synthesis or destruction of a certain chloroplast factor, as shown in the study of the uORF participation in the repression of the transcription factor AtHBI (Ribone et al., 2017). The question arises whether the matryoshka gene organization of the NbKPILP gene and the stress-regulating mechanism of its mRNA accumulation in leaves are widespread. Although the current assembly of N. benthamiana genome is incomplete, we found ~300,000 sORF in 41,354 transcripts of N. benthamiana according to the fifth version of transcriptome (Nakasugi et al., 2014), using the following criteria of a matryoshka gene organization: (a) mRNA contains nested short ORF (translated products between 10 and 100 amino acids), (b) sORF is translatable according to the sORFfinder prediction (Hanada et al., 2010), and (c) AUG codon of the sORF is in favorable Kozak’s context (consensus aMNauggG). It turned out that 1,409 ANRFs fall on 1,332 unique transcripts, in which 95% contain single ANRF, 4.4%—2 ANRFs and 0.6%—3 or 4 ANRFs. However, the proportion of ANRF among transcripts with different and identical levels of expression in roots and leaves was practically the same. Thus, the ANRF mechanism is likely to be used mainly for specific genes and tasks.
**AUTHOR CONTRIBUTIONS**

YD and TK: conceived of the hypotheses and designed the experiments; ES: performed most of the experiments; TK, AS, and NE: performed some experiments; YD, ES, TK, and AS: evaluated the data and drafted the outline of the manuscript; YD, ES, and TK revised and finalized the manuscript; All the authors read and approved the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2017.02137/full#supplementary-material

**Figure S1** | Molecular phylogenetic analysis of KPIs and KPILPs with highest sequence identity to NbKPILP (see Table S3) calculated from the amino acid sequences by the maximum likelihood method and based on the JTT matrix-based model (Jones et al., 1992). The evolutionary analysis was conducted in MEGA7.

**Figure S2** | The NbKPILP mRNA accumulation in N. benthamiana. (A) The NbKPILP mRNA accumulation in N. benthamiana organs according to the N. benthamiana Gene Expression Atlas programme (http://setappsi02.qut.edu.au/atlas/REX6.php); (B) Pictures of different ages of N. benthamiana plants isolated from soil. (C) The NbKPILP mRNA accumulation in N. benthamiana leaves and roots. The plants of different ages ranging from 2-week-old seedlings to 20-week-old flowering plants shown in (B), were used to analyse NbKPILP mRNA by qRT-PCR. The statistical significance of the difference in NbKPILP mRNA...
content between plants of different ages (5, 8, and 20 weeks) and seedlings (2 weeks) is shown.

**Figure S3** | Nucleotide sequence alignment of N. benthamiana Kunitz peptidase inhibitor-like (NbkPIL) mRNA (EMBL ID FN687760) with other homologous Solanaceae mRNAs: SmkPIL, Solanum melongena KPI (Sol genomics sequence ID Sme2.5_02047.1_g000091.1) NKkPIL (Nicotiana tabacum KPI, Sol genomics sequence ID NtNt-BK-AW01-S1966), CaPIL (Capsicum annuum KPI), sequence ID Ntb-BK-AW01-S1966), StkPIL, Solanum tuberosum KPI (EMBL ID XP_006353918), SkPIL, Solanum lycopersicum KPI (EMBL ID XM_004235444) and NgPIL, Nicotiana glutinosa biotic cell death-associated protein (EMBL ID AF208022). The polyurine block and ANRF are marked by red and yellow, respectively. Asterisks mark the nucleotides which are the same for all sequences. Hyphens represent sequence gaps. The alignment was executed by the Clustal Omega programme (http://www.ebi.ac.uk/Tools/msa/clustalo/).

**Figure S4** | Amino acid sequence alignment of polypeptides encoded by ANRFs.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.