Effect of 42 amino acid long amyloid-β peptides on Arabidopsis plants

HanGyeol Lee · Ji Woo Kim · Sangyun Jeong · Jungeun An · Young-Cheon Kim · Hojin Ryu · Jeong Hwan Lee

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Abstract Although the evolution of Arabidopsis thaliana and humans diverged approximately 1.6 billion years ago, recent studies have demonstrated that protein function and cellular processes involved in disease response remain remarkably conserved. Particularly, γ-secretase, a multisubunit protein complex that participates in intramembrane proteolysis (RIP) regulation, is also known to mediate the cleavage of more than 80 substrates including the amyloid precursor protein (APP) and the Notch receptor. Although the genes (PS1/2, APH-1, PEN-2, and NCT) coding for the γ-secretase complex components are present in plant genomes, their function remains largely uncharacterized. Given that the deposition of 42 amino acid long amyloid-β peptides (hAβ_42) is thought to be one of the main causes of Alzheimer’s disease, we aimed to examine the physiological effects of hAβ_42 peptides on plants. Interestingly, we found that Arabidopsis protoplast death increased after 24 h of exposure to 3 or 5 µM hAβ_42 peptides. Furthermore, transgenic Arabidopsis plants overexpressing the hAβ_42 gene exhibited changes in primary root length and silique phyllotaxy. Taken together, our results demonstrate that hAβ_42 peptides, a metazoan protein, significantly affect Arabidopsis protoplast viability and plant morphology.

Keywords Alzheimer’s disease, Amyloid-β peptide, Arabidopsis mesophyll protoplasts, γ-secretase, Transgenic plants

Introduction

Large functional genomics initiatives have revealed that many human disorders are affected by multiple genes and their genetic and molecular interactions with each other and the environment. Thus, the use of various model systems such as human and mammalian cell lines, yeast (i.e., Saccharomyces cerevisiae), as well as other organisms such as Caenorhabditis elegans and Drosophila melanogaster contributes to important discoveries within human disease research and the design of new diagnostic tools and medical treatment strategies (Xu and Moller 2011).

Recent reports have shown that Arabidopsis thaliana known as a model organism for plant biology is a valuable model system to study molecular mechanisms underlying human disease states (Hays 2002). According to the comparison between Arabidopsis and human genome sequences, Arabidopsis genome encodes many orthologues of human proteins albeit Arabidopsis and humans diverged 1.6 billion years ago (Xu and Moller 2011). For example, TAIR WU-BLAS tool (http://www.arabidopsis.org) analysis showed that 71% of genes involved in neurological human diseases have Arabidopsis orthologues with high E-value cutoffs. Furthermore, Arabidopsis has some advantages for human disease research: (i) Simple cultivation and maintenance with low infrastructure and operating costs, (ii) Fast and simple transformation techniques for transgenic analyses, (iii) The availability of large mutants and genomic resources, and (iv) few ethical restrictions (Xu and Moller 2010).

Alzheimer’s disease (AD) is the most common age-related neurodegenerative disorder of the central nervous system (Mattson 2004), which is characterized by the deposition of aggregation-prone amyloid-β peptides (Aβs) as the degraded products of the amyloid precursor protein (APP) and neurofibrillary tangles in the brain (Selkoe 1998). In humans, presenilin (PS) was identified from the genetic screens of patients suffering from AD and mutations in PS1 or PS2 led to improper cleavage of APP, which resulting in Aβs accumulation and progressive neurodegeneration (Sherrington et al. 1995). PS as a catalytic core protein is a component of γ-secretase, which is implicated in the process of 

H. G. Lee · J. W. Kim · S. Jeong · J. An · Y.-C. Kim · J. H. Lee (✉)
Division of Life Sciences, Jeonbuk National University, 567 Baekje-dae-ro, Deokjin-gu, Jeonju, 54896, Republic of Korea
e-mail: jhwanlee90@jbnu.ac.kr

Hojin Ryu (✉)
Department of Biology, Chungbuk National University, Cheongju 28644, Republic of Korea
e-mail: hjryu96@chungbuk.ac.kr

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of regulated intramembrane proteolysis (RIP) (Sannerud and Annaert 2009). Besides PS, nicastrin (NCT), presenilin enhancer 2 (PEN-2), and anterior pharynx-defective 1 (APH-1) as other constituents of γ-secretase are required for complex assembly, stabilization, trafficking, and substrate recognition (Parks and Curtis 2007).

APP as one of more than 80 substrates for γ-secretase is a metazoan protein, which is only present in genomes of multicellular animals (Haapasalo and Kovacs 2011). However, the components of γ-secretase have been identified in both metazoan and plants (Grigorenko et al. 2002; Kimberly and Wolfe 2003; Moliaka et al. 2004; Ponting et al. 2002; Wigge and Weigel 2001). The moss Physcomitrella patens also harbors homologues of all γ-secretase subunits with PS present in one copy. A lesion in moss PS (PpPS) showed several pleiotropic defects such as abnormal growth pattern, impaired chloroplast movement, and decreased uptake of an endocytosis tracer (Khandelwal et al. 2007). The mutation in PS of Dictyostelium discoideum as a slime mould affected cell fate determination and the regulation of phagocytosis (McMains et al. 2010). In Arabidopsis as a higher plant, all γ-secretase subunits interacted with each other and colocalized in endomembrane system compartments (Smolarkiewicz et al. 2014). These data suggest that γ-secretase is evolutionarily conserved and its role may be extended beyond RIP found from animal studies.

Here we presented the effect of 42 amino acids long-human amyloid-β peptide (hAβ42) on Arabidopsis protoplasts, tissues, and transgenic plants.

**Materials and Methods**

**Plant materials and growth conditions**

The wild-type [ecotype Columbia (Col-0)] and the transgenic Arabidopsis plants were grown in soil or a murashige and skoog (MS) medium at 23°C under long-day (LD) conditions (16 h light/8 h dark) at a light intensity of 120 mmol·m⁻²·s⁻¹.

For treatment of 42 amino acids long-human amyloid-β peptide (hAβ42) at cellular level, we followed the procedures for isolation of Arabidopsis mesophyll protoplasts, as previously described (Lee et al. 2017; Yoo et al. 2007). Isolated protoplasts (2 × 10⁶) were treated with 3 and 5 mM hAβ42 monomer or DMSO, and then the percentage of dead cells at indicated times was determined by staining protoplasts with Evans blue dye (Sigma-Aldrich), which was added to the samples to a final concentration of 0.04 % (Danon et al. 2005; Wright et al. 2000). The number of stained cells was measured using a light microscope (Zeiss, Oberkochen, Germany). For treatment of hAβ42 at tissue level, Arabidopsis plants were grown on soil or MS medium under LD conditions at 23°C. 10-d-old plants were treated with 3 mM hAβ42 monomer or Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, Missouri, USA), as previously described (Kim et al. 2017), and then hAβ42-treated plants were observed after 2 and 4 days under a light microscope (Nikon, Tokyo, Japan).

**Preparation of hAβ42 monomer**

The procedures for were previously described (Dahlgren et al. 2002; Kook et al. 2012). hAβ42 peptide was dissolved at a concentration of 1 mg·ml⁻¹ in 3,3,3-Hexafluoro-2-propanol (HFIP) (Sigma-Aldrich). HFIP was evaporated using Speed Vacuum concentrator and lyophilized peptide was resolved in 10 ml DMSO. After calculation of the molar concentration, aliquots of 1 mM lyophilized hAβ42 monomer were stored at -80°C until use.

**Generation of transgenic Arabidopsis plants**

For p35S::hAβ42 construct, the region of human Aβ42 was polymerase chain reaction (PCR)-amplified using plasmid DNA template with the gene encoding human amyloid precursor protein (hAPP) [gifted form Dr. Jeong (Jeonbuk National University, South Korea) and the resulting products were cloned into the pCHF3 vector harboring cauliflower mosaic virus (CaMV) 35S promoter. The resulting recombinant plasmid was sequenced to verify the absence of the PCR errors during amplification. Oligonucleotide primers used for cloning were as follows: 5'-ACGAGCTCGTACCCGGGATGCTGCCCGGTTTGGC -3' and 5'-CTCTAGAGGATCCCCGGGCGTATGACAACACCGCCC -3'. This construct was transformed into Agrobacterium tumefaciens strain GV3101 and then infiltrated into the wild-type (Col-0) plants. Transgenic plants were generated using the floral dip method with minor modifications (Weigel and Glazebrook 2002). The transgenic seedlings were first selected using kanamycin for a pCHF3 vector and then verified using PCR-based DNA genotyping. At least 30 ~ 40 T₁ seedlings were analyzed for the construct.

To examine the expression of hAβ42 in the transgenic Arabidopsis plants, we performed semi-quantitative reverse transcription (RT)-PCR, as described previously (Lee et al. 2007). Total RNA was extracted from 8-d-old seedlings using a RiboEx Total RNA kit (GeneAll, Seoul, Korea), and
first-strand complementary DNA (cDNA) was synthesized from 1 μg of total RNA, in accordance with the manufacturer’s instructions (GeneAll). The following oligonucleotides were employed as gene-specific primers in the RT-PCR analyses: for hAβ42, 5'-GAGCTCATGCTGCCCGTGTTGCACT -3' and 5'-TCTAGACGCTATGACAACCGCACC ACC-3'; for AtPP2AA3, 5'-GCGGTTGTGGAGAACATGATACG-3' and 5'-GAACCAAACACAATTCGTTGCTG-3'. AtPP2AA3 was used as a reference gene.

Results and Discussion

The treatment of hAβ42 monomer in Arabidopsis mesophyll protoplasts affects the viability of the cells

Because the accumulation of aggregated form of Aβ42 in the brain leads to mitochondrial dysfunction and elevated reactive oxygen species (ROS), consequently resulting in cell death (Reddy and Beal 2008), we investigated whether hAβ42 monomer affects the viability of plant cells. Mesophyll protoplasts were isolated from 10-d-old Arabidopsis wild-type (Col-0) plants grown at 23°C under LD conditions and 2 × 10^4 protoplasts were treated with 3 or 5 mM hAβ42 monomer at 1, 2, 4, 8, and 24 h under continuous light conditions. The protoplasts treated with DMSO were used as a control. Because dead cells were specifically stained with Evans blue dye (Danon et al. 2005; Wright et al. 2000), we stained 3 or 5 mM hAβ42 monomer-treated protoplasts after 24 h with Evans blue dye. This indicates that dead cells were increased in the protoplasts treated with 3 or 5 mM hAβ42 monomer, compared with DMSO-incubated protoplasts (Fig. 1a). We also calculated the percentage of dead cells at 0, 1, 2, 4, 8, and 24 h under a light microscope. The percentage of dead cells in protoplasts treated with hAβ42 monomer gradually increased and reached approximately 60% after 24 h (Fig. 1b). The portion of dead cells was nearly similar irrespective of treatment of 3 or 5 mM hAβ42 monomer. In contrast, the percentage of dead cells in DMSO-treated mesophyll protoplasts remained almost constant until 8 h and showed approximately 40% after 24 h (Fig. 1b). These results indicated that hAβ42 monomer reduces the viability of Arabidopsis mesophyll protoplasts.

The treatment of hAβ42 monomer does not cause any morphological changes in Arabidopsis young seedlings

Because hAβ42 monomer affects the viability of Arabidopsis mesophyll protoplasts at cellular level (Fig. 1), we next examined the effect of hAβ42 monomer at plant tissue levels. Then, we sowed Arabidopsis wild-type (Col-0) seeds in solid MS medium, transferred 10-d-old seedlings into 6-well plates containing 1 mL of liquid MS medium with 3 mM hAβ42 monomer or DMSO, and observed the morphological changes of seedlings after 2 and 4 days. The 3 mM hAβ42 monomer-treated seedlings after 2 and 4 days did not show any defects of morphology such as leaves, hypocotyls, and roots (Fig. 2). This result indicated that hAβ42 monomer does not affect the growth of young seedlings at the tissue level. However, we cannot dismiss the possibility that young seedlings may be more resistant to physical and chemical treatment than protoplasts.
In order to investigate the effect of the hAβ42 silique phyllotaxy plants leads to morphological changes in root length and that three independent lengths were quantified 8 days after germination. We found the seedlings were grown on MS medium and primary root transgenic plants in the T

dopsis p35S::hAβ42 (Fig. 3a). To examine the morphological changes of the transgenic plants with a single under the control of a p35S

overexpression of hAβ42 in transgenic Arabidopsis plants leads to morphological changes in root length and silique phyllotaxy.

The overexpression of hAβ42 in transgenic Arabidopsis plants leads to morphological changes in root length and silique phyllotaxy.

In order to investigate the effect of the hAβ42 overexpression in Arabidopsis plants, we expressed the region of hAβ42 under the control of a 35S promoter (p35S::hAβ42) in Arabidopsis wild-type (Col-0) plants. We obtained 37 independent transgenic plants in the T1 generation from DNA genotyping (Fig. 3a). To examine the morphological changes of the p35S::hAβ42 plants in more detail, we generated the transgenic plants with a single p35S::hAβ42 insertion in the T1 generation and confirmed a higher expression level of introduced hAβ42 gene (Fig. 3b). Firstly, we compared root growth phenotypes of wild-type (Col-0) and p35S::hAβ42 plants. The seedlings were grown on MS medium and primary root lengths were quantified 8 days after germination. We found that three independent p35S::hAβ42 lines had long primary roots than wild-type (Col-0) seedlings (Fig. 3c). The degree of primary root lengths was correlated with the expression levels of hAβ42 gene in independent p35S::hAβ42 lines. For instance, a p35S::hAβ42 line (#16) showing strong hAβ42 gene expression had more longer primary roots than p35S::hAβ42 line (#8). Secondly, we measured the flowering time in six independent p35S::hAβ42 lines under long-day (LD) conditions at 23°C. We found that the flowering time of four independent p35S::hAβ42 lines (#14, #22, #26, and #36) was unchanged, whereas two independent lines (#8 and #16) has altered flowering time (Fig. 3d and e). However, the degree of flowering time was not correlated with the expression levels of hAβ42 gene in independent p35S::hAβ42 lines.

Lastly, we detected abnormal phyllotaxy in the inflorescence of two independent p35S::hAβ42 lines (#16 and #22) (Fig. 3f), which showing that strong hAβ42 gene expression in p35S::hAβ42 lines affected the degree of defects in phyllotaxy. These results indicated that the increased hAβ42 expression affects morphological changes in root length and silique phyllotaxy in the p35S::hAβ42 plants.

Conclusions

Here we provide the first data on the effect of hAβ42 monomer as a metazoan protein in angiosperms. In a series of experiments, we showed that the viability of cells was reduced in hAβ42 monomer-treated Arabidopsis mesophyll protoplasts (Fig. 1), suggesting that hAβ42 monomer may lead to cell death. Because the protoplast system is known as a useful tool that allows an easy uptake of bioactive agents and a concise quantification of cell death response (Yoo et al. 2007), we will further investigate whether the features of the cellular destruction such as DNA cleavage, the activation of caspase-like molecules, and the release of mitochondrial components are observed in hAβ42 monomer-treated protoplasts (Reape and McCabe 2008). Although treatment of hAβ42 monomer did not affect the growth of young seedlings at the tissue level, hAβ42 gene expression in the transgenic Arabidopsis plants caused the morphological changes in primary root length and silique phyllotaxy (Fig. 3). A recent report have shown that Arabidopsis ps1 ps2 double mutants have accelerated chlorosis in the dark (Smolarkiewicz et al. 2014). Because darkness and starvation induce senescence and autophagy in plants (Sobieszczuk-Nowicka et al. 2018), we will further examine the effect of prolonged darkness treatment on hAβ42 monomer-treated Arabidopsis seedlings and p35S::hAβ42 plants.
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