Seed-Specific Expression of Apolipoprotein A-I $_{Milano}$ Dimer in Rice (*Oryza Sativa* L.) Transgenic Lines

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Abstract

Apolipoprotein A-I $_{Milano}$ (ApoA-I_M) has been shown to significantly reduce coronary atherosclerotic plaques. However, the preparation of cost-effective pharmaceutical formulations of Apo A-I $_{Milano}$ can be limited by the high cost and difficulty of purifying the protein and producing the highly effective dimeric form. The aim of this study was the characterization of rice plants expressing the ApoA-I $_{Milano}$ protein primarily in a dimeric form, specifically in the seeds. The seed-specific 13 kDa rice-prolamin promoter drives the expression of ApoA-I $_{Milano}$ to different caryopsis tissues such as seed coat, aleurone cells, and endosperm, particularly into amyloplast and storage vacuoles. A plant-based ApoA-I $_{Milano}$ production system offers numerous advantages over current production systems, including direct production of the most therapeutically effective dimeric ApoA-I $_{Milano}$ forms, long-term storage of protein in seeds, and ease of protein production by simply growing plants. Therefore, seeds could provide a cost-effective source of ApoA-I $_{Milano}$ for use as a therapeutic.

Introduction

Apolipoprotein A-I (ApoA-I) is a component of high-density lipoprotein (HDL). It constitutes approximately 70% of HDL. HDL is a molecule that transports cholesterol and certain fats called phospholipids through the bloodstream from the body's tissues to the liver. Once in the liver, cholesterol and phospholipids are redistributed to the other tissues or removed from the body[1]. HDL is often called "good cholesterol" because high levels of this substance reduce the chance of developing heart and cardiovascular disease[2]. ApoA-I is the subject of intense research for its anti-atherogenic properties. Infusion of ApoA-I mimetic peptides has been shown to stimulate cholesterol efflux from tissues into plasma[3].

The naturally occurring mutant ApoA-I $_{Milano}$ (ApoA-I_M) is characterized by the presence of a cysteine residue in position 173 (R173C) that promotes disulphide bonds and the subsequent formation of dimers; dimers increase protein stability[4] and promote greater interaction with lipids and with the enzyme lecithin-cholesterol acyltransferase (LCAT), thereby increasing the therapeutic effect of the protein[5]. Dimers of the ApoA-I_M variant can form HDL particles with the same efficiency as ApoA-I[6]. Several studies[7] indicate that 'HDL Therapy' with infusions of a formulation (ETC-216: synthetic HDL phospholipids complex) of ApoA-I_M significantly reduces coronary atherosclerotic plaques.

Although the therapeutic effects of apolipoproteins have been proven, there are numerous difficulties in their preparation as pharmaceuticals, mainly related to the high cost of production.

The production of recombinant proteins in bacterial systems is attractive for their capacity to produce high quantities but also shows difficulties such as the presence of undesirable affinity signals at the end of the purification[8]. Further, the endotoxins of *E. coli* are known to form strong complexes with apolipoproteins[9]. The elimination of these toxins for pharmaceutical products is necessary and technically possible but requires complex and costly methods[10]. The production of the more

therapeutically effective dimer form of Apo A-I_M can be accomplished by purification of monomers from *E. coli*, as described in patent US6617134B1 with a subsequent dimerization process, however, this is a timely and expensive procedure that is not without risk.

The therapeutic use of apolipoproteins is therefore limited by the scarcity of methods to prepare the protein in a sufficient quantity, and a form optimized for administration. In particular, the production of Apo A-I with recombinant methods has been shown to be very difficult due to its amphiphilic character, autoaggregation, and degradation[11]. Thus, there is a need for a system of production or expression that easily, safely, and cost-efficiently produces apolipoproteins in dimeric form.

The ability to introduce foreign genes into plant species by techniques such as agroinfection or by direct gene transfer has opened up the possibility of using transgenic plants as host organisms for the production of heterologous proteins[12]. Plants have several advantages that make them more attractive for this purpose: i) they represent an economic system, compared to mammalian cell cultures and microbial fermentation, ii) the expression of heterologous proteins can be induced in specific organs as seeds, tubers, etc, where proteins are more stable [13,14], iii) therapeutic proteins derived from plants, whether purified or not, are less likely to be contaminated with human pathogenic microorganisms than those derived from animal cells because plants are not hosts for human or animal infectious agents[15,16]. iv) Plants can perform most of the post-translational modifications (glycosylation, prenylation) required for protein stability, and bioactivity in the same way as other higher eukaryotes17; v) proteins can be expressed in the edible organs of plants in order to be consumed raw as an edible vaccine eliminating the need for downstream processes[17,18]. Downstream processing when required is easy and less expensive, particularly when proteins are expressed in specific organs like seeds.

A previous study showed that rice plants transformed for Apo A-I_M expression provide rice milk with valuable therapeutic effects in protecting mice from atherosclerotic plaques, suggesting the use of these molecules in cardiovascular diseases[19].

Apo A-I_M transformed rice plants have been further characterized in the present work and the localization of the protein was studied in two different stages of seed ripening in transgenic plants.

Results

Molecular analysis of putative transformed plants

The rice expression vector pCambia-PROL-ApoA-I_M (**Figure 1a**) containing ApoA-I_M gene under the control of the 13 KDa seed promoter was developed. In order to confirm the correct insertion of the ApoA-I_M gene and promoter in the expression vector pCAMBIA-PROL-ApoA-I_M, the plasmid was digested with different restriction enzymes and subjected to electrophoresis on an agarose gel. The digested fragments corresponding to the expected size according to the map of recombinant plasmid (**Figure 1b**). The fusion from promoter and gene and the correct frame of translation was confirmed by sequencing. A total of

100 calli derived from mature embryos of Rosa Marchetti were transformed by agroinfection and approximately 32 independent hygromycin-resistant T0 transformed plants were obtained and grown under greenhouse conditions and evaluated for morphology and fertility. Considering the seeds production, about 93% of the transformed plants were fertile and presented the same morphological aspects of the controls (untransformed plants).

We analysed all of the independently derived transgenic plants by the PCR for the presence of the ApoA-I_M gene using Apo-fw and Apo-rv specific primers (**Table 1**). Plants containing the Apo A-I_M gene were identified by a band corresponding to the expected PCR product size of 732 bp (**Supplementary Figure. S1**). Approximately 78% of rice plants resulted transformed.

The status of the transgenic plants was characterized by Southern Blot analysis in detail, and the copy number of the ApoA-I_M gene in transgenic plants was estimated. We analysed 16 independently derived T0 transgenic plants by Southern Blot. Unique and complex hybridization patterns were revealed, indicating that these plants were indeed originated from independents events (**Figure 2**). All the 16 transgenic plants analysed showed positive hybridization and most of the hybridization bands were between 3 and 8 Kb. Most of the transgenic plants carried more copies of the transgenes ApoA-I_M, only 4 plants carried one copy of the transgenic gene (**Figure 2** asterisks). No hybridization bands were found in the untransformed rice plants as control. Southern blot analysis confirmed that the Apo A-I_M gene had stably integrated into the genome of the transgenic plants.

Segregation for hygromycin resistance of plant numbers 3, 15, 23, and 25 followed the classic Mendelian inheritance 3:1(resistant to sensitive) confirming a single insertion site, as shown by a single band (**Figure 2** asterisks).

Expression analysis of Apo AIM in transgenic plants

All T1 seeds from PCR positive lines were analysed for the protein expression in reducing and non-reducing conditions (+/- β-mercaptoethanol) to visualize respectively monomer and dimer of APOA-I_M production. Approximately 64% of PCR positive lines produce the monomer ApoA-I_M. Using ApoA-I antiserum, one immunoreactive polypeptide with a molecular weight of 28 KDa was detected. The 75% of T1 seeds producing ApoAI_M monomer in non-reducing conditions confirm the ApoA-I_M dimer presence. Using ApoA-I antiserum, immunoreactive polypeptides with apparent weights of 28 KDa and a doublet at about 56 KDa were detected (**Supplementary Figure S2**). The relative expression level of monomeric and/or dimeric forms of the Apo A-I M protein in seed extracts differs in analysed plant lines. The ApoA-I_M quantity (monomeric and dimeric form) was quantified by ELISA IC assay. The expression level was resumed in **Table 2**. At the same time, the seeds of T2 plants were analysed to verifier if silencing phenomes were possible.

The number of gene copies of the introduced ApoA-I_M genes could not be strictly correlated with expression levels in the same plants, suggesting that the variation in expression resulted from other

factors, such as chromosomal position effects of the integrated genes in the *Oryza* genome.

Temporal expression of the prolamin promoter was first proved by differential display experiments considering different days after flowering (DAF) stages in rice seeds (*Oryza sativa* cv. Ariete) (Availability of materials and data).

Total proteins were isolated from ApoA-I_M transgenic seeds at different ripening stages (4, 8, 12, 16, 20, and 25 DAF), loaded on 15% acrylamide gel in denaturing conditions. After blotting on nitrocellulose membrane, the hybridization with antiserum ApoA-I was performed. As shown in **fig. 3a**, ApoA-I_M protein was detected early, 8 DAF, and rapidly reached the plateau between 12 and 16 DAF, remaining at the maximal level during the following ripening stages.

At the same time, western blotting was performed on different tissues of rice to the verifier the seed expression. Protein extracted from seeds, leaf, culm, and root from ApoA-I_M transgenic plants was blotted and hybridized at the same condition. As shown in **fig. 3b**, ApoA-I_M signal was detected only in the seeds; leaf, culm, and roots showed absence of positive hybridization maybe after a prolonged exposition.

Immunolocalization of ApoAI $_M$ in seed tissues

Localization of ApoAI $_M$ was further analysed by immunocytochemical and immunogold observations. Caryopsis at two different developmental stages (called I and II respectively) were collected in the mid maturation phase (around 25 DAF) from the same panicle and observed by fluorescence and electron microscope.

In caryopsis I (**Figure 4**) the pericarp, seed coat, and nucellus were clearly observed (**Figure 4b**) and only a weak autofluorescence was observed in these tissues after anti-ApoAI $_M$ antibody treatment (**Figure 4a, b**). On the contrary, a high fluorescence was observed in the endosperm cells while ApoAI $_M$ was excluded from aleurons cells (**Figure 4a-d**). In the endosperm cells, ApoAI $_M$ was localized into amyloplasts (**Figure 4c, d**), particularly in the stroma, surrounding starch granules which, in turn, appeared dark (**Figure 4c**; arrows). Negative controls showed only a faint autofluorescence and no cross-reaction with seed tissues (**Figure 4e, f**).

The pattern of ApoAI$_M$ distribution changed in caryopsis II (**Figure 5**) in which the pericarp appeared thinner due to the flattening of mesocarp and seed coat cells (**Figure 5a-f**). Nucellus appeared also compressed by the endosperm growth and hardly recognizable (**Figure 5a-f**). Immunofluorescence analyses showed that ApoA-I_M was localized not only in endosperm cells as in seeds I, but also in seed coat and aleurone cells (**Figure 5 a, c**), suggesting that during seed ripening the expression/distribution of this protein changed. High fluorescence was observed in the seed coat cell cytoplasm (**Figure 5a, c, i**). ApoA-I_M is localized in the cell wall of pericarp cells, close to seed coat while, in the cytoplasm, autofluorescence occurred as observed also in the negative control (compare **Figure 5 c**, **d**, and **e**, **f**). In the

endosperm, round organelles similar to amyloplasts were fluorescent and, interestingly, ApoA-I_M was also localized in the cell wall and in small organelles of the aleurone cells (**Figure 5c**, **d**, **g**, **h**; arrows).

To better investigate the ApoA-I_M localization inside cells, immunogold analyses were performed. Different anti-ApoAI_M antibody concentrations were used to exclude aspecific reactions with other cell components and the observations **confirmed** the presence of ApoAI_M in the endosperm, aleurone cells, and seed coat (**Figure 6**). In the seed coat, ApoA-I_M was localized in the primary cell wall (**Figure 6 a**) and into chloroplasts, particularly at the grana (**Figure 6 b**, **c**). Aleurone cells were not completely differentiated and some starch grains were also observed (**Figure 6 d**, **f**). However, numerous storage vacuoles, precursors of protein bodies (PBs) were stained with ApoA-I_M antibodies which were observed as dark spots in the peripheral area of storage vacuoles (**Figure 6 d**, **f**). ApoA-I_M was also observed in vesicles associated with dictyiosomes (**Figure 6 e**) and in the cell wall which showed numerous plasmodesmata (**Figure 6 f**). Some dark spots were also observed in the cytoplasm (**Figure 6f**), suggesting the presence of cytoplasmic recombinant protein, In the endosperm, which has not already completed its development and Programmed cell death, cells showed both storage vacuoles and numerous amyloplasts (**Figure 6 g, h**). Amyloplasts appeared bigger compared to those observed in the aleurone cells and contained several starch granules (**Figure 6 g, h**). In endosperm, ApoA-I_M localized both in storage vacuoles and in amyloplasts (**Figure 6 g, h**). Negative control did not show any staining (data not shown).

Discussion

HDL and its major protein ApoA-I are protective against atherosclerosis through several mechanisms, including the ability to mediate reverse cholesterol transport. ApoA-I formulations and ApoA-I mimetic peptides demonstrated rapid effects on plaque regression and stabilization[3]. ApoA-I_M is a natural variant characterized by Dr. Cesare Sirtori in 1974 that differs from native protein in that cysteine is substituted at position 173 for arginine allowing disulphide-linker dimer formation. The variant Milano was subjected to numerous studies and interesting clinical trials[20] that demonstrated its important atherogenic properties. Recombinant expression of ApoA-I_M in different systems (e.g. bacteria, yeast, insect cells, and mammalian cells) has been achieved, even if with not high level of expression. Many different expression systems are available for the production of pharmaceutical proteins and the selection of the appropriate system depends on a variety of factors including the host's ability to express the desired product (e.g. processing and post-translational modification) and the complexity and scalability of the product. Bacterial expression systems are commonly used for therapeutical heterologous protein production as these systems provide an economical route for protein expression and require minimal technical expertise to set up a lab production system. Nevertheless, prokaryotic cells are not capable of the post-translational modifications for biological activity and in the Gram-negative bacterial systems, such as *E. coli*, endotoxin contamination can pose a significant problem in purification. On the contrary, eukaryotic cells, tissues, or organisms, available as expression hosts, can produce complex proteins with the correct folding and post-translational changes but they are expensive to operate, have a longer growth period,

and present contamination risks. Plants have been utilized for more than two decades for the production of many pharmaceuticals proteins and many encouraging results were obtained also in the field of edible vaccine. Many plant-based vaccines have been developed in different plant expression systems and evaluated against various life-threatening diseases.

In our study, the production of ApoA-I_M in rice offers an attractive alternative for efficient and inexpensive recombinant protein production while minimizing the risk for contamination by human pathogens.

In our study, the 13 KDa prolamin promotor allowed the seed-specific expression of the recombinant ApoA-I_M protein in transformed rice plants. Seeds have proven to be effective tools for recombinant protein production, including peptides or short and long polypeptides as well as complex like mammalian immunoglobulins[21]. The extraction and recovery of recombinant proteins from seeds is greatly assisted by their dormancy properties because this allows for the long-term stability of stored heterologous proteins and decoupling of processing from the growth and harvest cycles. Furthermore, the low water content reduces the possibility of microorganism's secondary contamination (e.g. bacteria and fungi), possessing possess more storage opportunities[22].

Moreover, ApoA-I_M was expressed in rice seeds in dimeric form and its activity was efficiently determined by Romano et al. (2018). In this study, the rice plants expressing APOA-I_M were further investigated, particularly seed expression and its localization at the cellular level.

We have obtained different lines of transgenic rice plants for ApoA-I_M. As typically reported in plants, transformed with binary vectors via Agrobacterium species, the integration level of the pCAMBIA-PROL-APOA-I_M construct was variable. A dissimilar pattern of hybridization products was obtained from different plants, suggesting different gene copy numbers and integration of the transgene into sites with variable transcription competencies. The absence of detectable PCR products in some plants, despite hygromycin selection, could reflect the development of antibiotic resistance, genetic instability after integration, and partial integration of expression vector[23].

The prolamins are important storage proteins in rice, and they are classified by size as 10, 13, and 16 KD[24]. A 13 KDa prolamin promoter used in this study, was demonstrated as an excellent candidate for the Apo A-I_M expression in rice for its seed-specificity and its stability.

According to data reported from the analysis of promoters useful for expression of recombinant proteins in seeds of rice plants[25], the expression of Apo A-I_M proteins in transgenic rice plants was specific for seed tissues; in fact, immunoblotting performed on leaves, roots, and stem protein extracts of positive plants did not reveal the presence of recombinant protein. The expression box 13KDa prolamin promoter in association with NOS terminator is sufficient for seed specificity; on the contrary, it has been reported that the 10kDa prolamin promoter is not completely effective for the seed-specific expression due to the association in the expression cassettes with NOS terminator[25].

The expression of Apo A-I_M protein follows a time course pattern during seed ripening; western blotting experiments revealed the ApoA-I_M protein accumulated early at 8 DAF and rapidly reached the maximum expression at 18 DAF (mid-maturation). The level of expression remained stable just at complete maturation of the seeds, according to Saito et al. (2012)[26].

The subcellular localization of a recombinant protein strongly influences its stability, accumulation, and ultimately the yield on recovery. Protein targeting also plays a key role in many types of post-translational modifications, among which glycosylation is the most relevant for molecular farming because the glycan chains can affect protein structure, biological function, and immunogenicity. Targeting to a specific compartment may therefore influence the interrelated processes of folding, assembly, and ultimate accumulation site (deposition), which have a direct impact on protein stability and yield. Seeds, therefore, offer several alternative subcellular destinations for recombinant proteins including Protein Bodies (PBs) derived from the reticulum endoplasmatic (ER), protein storage vacuoles (PSVs), starch granules, and the surface of oil bodies.

Immunocytochemical experiments showed a different localization of apolipoproteins in caryopsis at two different stages of ripening (mid maturation phase), from the same panicle. In the early stage, the Apo AI is expressed in the amyloplasts of endosperm cells. Later in addition to endosperm, the recombinant protein also localized in pericarp and aleurone cells. Electron microscopy observation on caryopsis in a later stage showed the distribution of ApoA-I in different cell organelles; as reported for 13 kDa prolamins[26], the recombinant proteins were observed in PBs. The presence of Apo AI occurred in the middle and surrounding layer of PBs. In fact, the 10 KDa prolamine was mainly detected in the core of PBs, whereas the 13kDa and 16 KDa proteins were detected in rings surrounding the 10 kDa prolamin-rich core.

The distribution pattern of ApoA-I_M in mid and external layers of PBs, reflects the expression timing of 13 KDa prolamin promoter. Indeed, the 10 KDa prolamin accumulated in the seed very early, from 4 DAF, on the contrary, the accumulation of 13 KDa prolamin begins later[26].

Interestingly, the ApoA-I_M was also observed in plastids in both stages of seed ripening. Moreover, in the late phase, ApoA-I_M is present in the cell wall and as a soluble protein in the cytoplasm.

These apparent mislocalizations of ApoA-I_M with respect to endogenous seed reserve proteins suggests that protein folding in rough endoplasmic reticulum (RER) or post-translational modifications in Golgi apparatus could be responsible for changes in Post-Golgi trafficking, leading to secretion of recombinant proteins into cell wall instead of to storage vacuoles (REFs). Intriguingly was observed in the cytoplasm and within plasmodesmata, suggesting that mRNAs for ApoA-I_M could be in part translated by cytoplasmic ribosomes instead ribosomes localized on RER and that the recombinant protein could be transferred among cells by symplastic route.

ApoA-I_M accumulated in the rice seeds in variable quantity, maximum recovery visualized was 8,2 mg/100g of rice seeds. ApoA-I production in *E.coli* recovered in literature was from 0,1 mg/L to 5 mg/L[27,28], but in some cases, the ApoA-I protein accumulated in inclusion bodies and required a refolding after purification. Nykiforuk et al. (2011)[29] expressed ApoA-I_M in a fusion protein in transgenic safflower seeds in very high quantity, but the ApoA-I_M rice offers some advantages as an inexpensive and a large production of edible materials ready to use.

Obtained transformed plants offer a promising option for large-scale APOA-I_M production. Further investigations are required in order to better establish its effect after oral administration by edible transformed rice.

Materials And Methods

Expression vector

The 13 KDa seed-specific promoter together with relative 5' UTR and transit peptide sequence (NCBI acc. N° D63901) was amplified from *Oryza sativa* var. Ariete using a forward and reverse primer (PROL-fw and PROL-rv, **table 1**). The 729 bp promoter fragment was inserted in pGEM-T (Promega, Madison WI USA), and the accuracy of the amplified promoter region was verified by DNA sequencing[30]. A *PstI* and *XbaI* site was inserted respectively in the forward and reverse primer to ease the subsequent DNA cloning in a plant expression vector (pCAMBIA1302, NCBI Acc. N° AF234298) in *PstI* and *XbaI*. The cDNA of mature ApoA-I_M obtained according to Romano et al.(2018)[19] was put under the control of the rice 13 KDa prolamin promoter and cloned respectively in *BamHI* and *SacI* (blunted) **(Figure 1a).**

Rice transformation and analysis of transgenic plants.

Embryonic calli derived from mature zygotic embryos of the rice (*Oryza sativa* var. Rosa Marchetti) were inoculated with *Agrobacteriumtumefaciens* strain EHA105 transformed by electroporation with the plasmid pCAMBIA-PROL-ApoA-I_M. Callus and bacterial induction, transformation, selection, and regeneration of transgenic tissues were performed as described in Hiei et al. (1994)[31], with minor modifications. Putatively transformed (hygromycin-resistant) plants (T0 plants) were potted in peat and hardened in a greenhouse together with controls (WT rice) to produce T1 seeds. These T1 seeds were then subsequently planted to produce T1 plants and T2 seeds. During the entire experimental period, the plants were evaluated for the principal agronomic characteristic.

PCR Analysis. Before flowering, T0 plants were subjected to PCR analysis to verify the presence of the transgene. The PCR was done using DNA extracted[32] from leaves of the rice lines resistant to hygromycin, using the primers Apo-fw and Apo-rv (**Table 1**), which amplify the entire Apo A-I gene, visualized, in the case of successful transformation, by a fragment of 732 bp.

Southern Analysis. Independent transgenic rice plants expressing the ApoA-I_M gene were subjected to Southern Blot analysis to verify the copy number and insertions of the transgene present in each plant. The total genomic DNA was extracted from young leaves according to Doyle and Doyle (1987)[33]. Approximately 10 µg of DNA was digested with *XbaI* which cuts only once in pCAMBIA-PROL-ApoA-I_M, fractionated on a 0,8% (W/v) agarose gel, transferred to a positively charged nylon membrane, and hybridized with a probe corresponding to ApoA-I_M CDS labelled with the PCR DIG Probe Synthesis Kit (Roche Molecular Biochemicals Penzberg Germany) following the manufacture's instruction. The primer set used in PCR analysis was also used to generate the DIG-labelled probe. The positive control consisted of the vector (pGEMT-APOA-I_M) linearized with the same restriction enzyme. The detection was realized with CDP-Star® (GE Healthcare, Chicago Illinois USA) following the manufacturers' instructions.

Evaluation of protein expression

The seeds collected from the T0 and T1 plants PCR positive were then used to verify the expression by Western Analysis. Briefly, the total protein from rice seeds (100 mg) was extracted from the seeds using extraction buffer (50 mM Tris-HCl pH 8.0, 5 mM EDTA, 200 mM NaCl, 0.1% Triton X-100, 1mM PMSF) in ratio 1:10 (w/v), mixed with SDS-loading buffer (with or without β-mercaptoethanol, to discriminate from monomer/dimer respectively), subjected to electrophoretic separation on an acrylamide gel, and transferred using electroblotting (solution of 25 mM Tris, 192 mM glycine, 20% methanol, 30 V at 4°C overnight) to a nitrocellulose membrane, hybond ECL (GE Healthcare, Chigaco Illinois USA). The membrane with the bound protein was placed in a solution of PBS-T and 5% skim milk, agitated for 60 minutes, washed, then exposed to the primary antibody Anti ApoA-I Goat 1:5000 (Acris, San Diego CA, USA) and subsequently to the secondary antibody Anti-Goat peroxidase conjugate, 1:12.000 (Sigma Aldrich, St. Louis Missouri USA). The membrane was washed several times and placed in a chemiluminescent detection solution, ECL (GE Healthcare, Chicago Illinois USA).

Protein quantification in rice seeds. An indirect competitive ELISA (IC-ELISA) was used to detect ApoA-I_M protein in rice seeds. In particular, recombinant hApoA-I (Sigma Aldrich St. Louis Missouri USA) was coated (600ng/ml) onto a micro-well plate overnight at 4°C, after which the plate was washed three times with 0,01 M PBS (pH 7) and then blocked with 200 µl of 5%(W/V) BSA for 2 h at 37°C. The plate was then washed with 0,01 M PBS containing 0,05% (v/v) Tween 20 (PBST). 100 µl of goat polyclonal anti-ApoA-I antibody (Acris, San Diego CA USA) diluted 1:6000 was mixed with serial dilution (1:1, 1:10, and 1:50) of rice seeds protein extraction (100 µl). From each mixture, 100 µl solution was added to each well coated, and the plate was incubated at 37°C for 1 h. The plate was washed with PBST and then a 1:5000 diluted solution (100 µl) of anti-goat IgG-HRP antibody (Thermofisher Scientific, Waltham Massachusetts USA) was added to each well and incubated at 37°C for 1 h. After that, the plate was rewashed with PBST, and a 50 µl of TMB was added to each well followed by incubation at 37°C for 15 min. To stop the reaction, 150 µl of 0,4 N hydrochloric acid (HCl) was added to each well, and then absorbance was measured at 450 nm using an ELISA plate reader (BIORAD, Hercules California USA). Each experiment was performed

in triplicates. The standard curve using recombinant hAPOA-I (Sigma Aldrich, St. Louis Missouri USA) protein was constructed. **(Supplementary Figure S3)**

Immunofluorescence and Immunogold analyses

Rice seeds (mid-maturation) were collected, reduced in small pieces, and fixed in 50mM Hepes, pH 7,4, 2% formaldehyde, and 0,2% glutaraldehyde, overnight at 4°C. Samples were repeatedly rinsed in 50mM Hepes, pH 7.4, dehydrated with increasing concentrations of ethanol, and embedded in LR Gold resin (Sigma Aldrich St. Louis Missouri USA) at -20°C. Semi-fine sections (2µm) and ultra-thin sections (80 nm) were obtained using a Reichert Jung Ultracut E microtome. Ultra-thin sections were collected with nickel grids (Agar Scientific, Stansted United Kingdom) for immunogold experiments.

Immunofluorescence analyses

Semi-fine sections were hydrated with TBS (Tris-buffered saline, 20 mM TRIS, 150 mM NaCl pH 7.6) for 5 minutes, blocked with 1% BSA in TBS for 1 hour at room temperature, and then incubated with primary goat polyclonal anti-ApoA-I antibody (Acris, San Diego California USA) diluted 1:500 overnight at 4 °C. After three rinses in TBS, sections were incubated in the FITC-conjugated antigoat secondary antibody (Santa Cruz Biotechnology, Dallas Texas USA) at 1:200 in TBS for 2 hours at room temperature, rinsed three times with TBS, and mounted in Cityfluor. Negative controls were performed with only the secondary antibodies. Fluorescence observations were performed with a Leica DMRB microscope (set filter BP450-490, RKP 410, long pass 515). Images were acquired by a Leica MC170 HD camera.

Immunogold analyses

Ultra-thin sections were hydrated with TBS for few minutes, blocked in BSA 1% in TBS for 30 min at room temperature, and incubated with primary goat polyclonal anti-ApoA-I antibody (Acris, San Diego California USA) at different dilution (from 1:500 to 1:200) in TBS at room temperature for 2 h. After three rinses in TBS, sections were incubated with 20 nm gold-conjugated rabbit anti-goat secondary antibody (purchased by BBA International, USA) diluted 1:100 for 1h at room temperature, rinsed three times with TBS, and post-fixed with 1% glutaraldehyde 15 min at room temperature. Sections were that stained with Uranyl acetate 3% for 20 min in the dark, rinsed with distilled water, dried, and observed with an EFTEM LEO 912AB transmission electron microscope (Zeiss, Oberkochen Germany) working at 100 kV.

Declarations

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Additional Information

Correspondence and request of additional materials should be addressed to RS or MA.

All figures presented in the manuscript are original.

The rice was cultivated in confined conditions in the greenhouse facilities of 4LAB diagnostics. previously authorized for GM plants cultivation. The rice seeds under patent n° PCT-IB2006-054948 were conferred from 4LAB diagnostics (responsible of the deposit) only for research purposes.

Author contributions

SR: construction vector, molecular analysis, contribution to the discussion, and writing the paper. EO: conceptualization of microscopy approach, sample processing for light and electron microscopy, immunolabelling, observation of samples by fluorescence and electron microscope, writing results and discussion on immunolabelling, contribution to the general discussion of the paper. AM: conceptualization of microscopy approach, observation of samples by fluorescence microscope, contribution to the discussion on immunolabelling, contribution to the general discussion of the paper. NS: sample processing for light and electron microscopy observation (sectioning), contribution to the general discussion of the paper. LR: contribution to the discussion and to write the paper, designed the molecular analyses experiments, and project administration. MD analysing data and editing. KP: plant transformation. All the authors contributed to writing the manuscript and commented on it.

Competing interests

The authors declare no competing interests.

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Tables

Table 1: oligonucleotides used in this study for amplification ApoA-I_M gene, and 13 KDa prolamin promoter. Specific restriction site used for the cloning in the expression vector was underlined.

Primer name	Nucleotide Sequence	Ref Seq	Amplicon size (bp)	PCR cycle
	-	X00566	732	95°C x 3’
Apo-fw	**GGATCC**GATGAACCCCCCCAGAGCC			95°C x 45”
	-			58°C x 1’
Apo-rv	**GATATCTCA**CTGGGTGTTGAGCTTCTTAG			30x
				72°C x 1’
				72°C x 5’
	-	D63901	729	95°C x 3’
Prol-fw	**CTGCAG**GAATTCCTTCTACATCGGCTTAGG			95°C x 45”
	-			58°C x 1’
Prol-rv	**TCTAGA**CGCAGAGGCATTGCATGCAACAATAGC			30x
				72°C x 1’
				72°C x 5’

Table 2: T0 rice plants obtained from transformation, relative controls (PCR, Western blotting, and Sothern analysis), and ApoA-I_M quantification by indirect competitive ELISA.

ID	PCR analysis	WB reducing	WB No-reducing	Southern Insertion number	Expression level mg/100g of seed
1	+	+	-	4	0.87
2	+	+	-	2	0.96
3	+	+	-	1	0.90
4	+	+	+	2	8.20
5	-				
6	+	-			
7	+	+	+	3	7.80
8	-				
9	+	+	+	5	3.80
10	-				
11	+	+	+	5	2.00
12	+	+	+	3	7.50
13	+	-			
14	+	+	+	2	3.18
15	+	+	+	1	4.50
16	-				
17	-				
18	+	-			
19	+	-			
20	+	+	+	4	4.00
21	+	-			
22	+	-			
23	+	+	+	1	5.80
24	+	+	+	4	6.75
25	+	+	-	1	1.52
26	+	-			
27	+	+	+	2	8.20

Figures

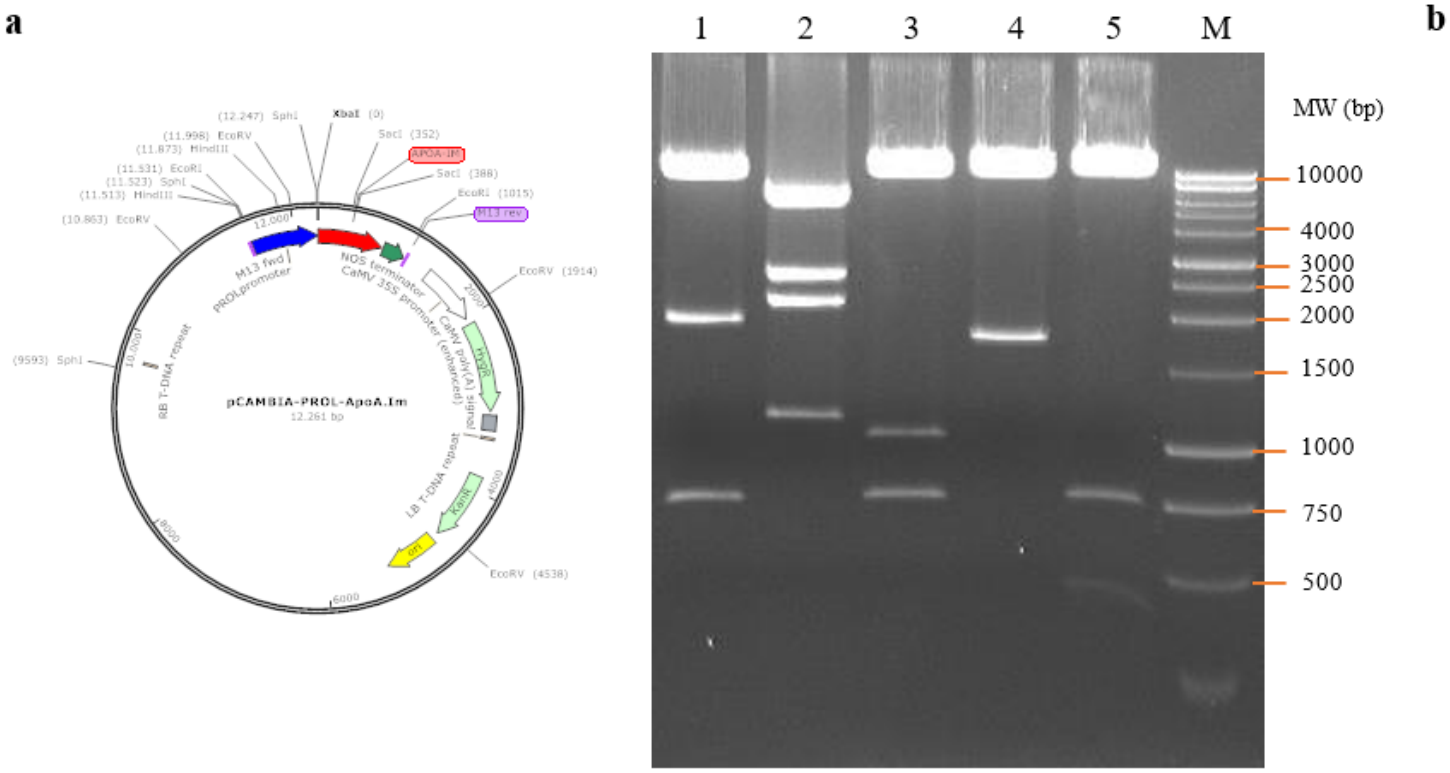

Figure 1

a) Schematic diagram of expression vector pCambia-PROL-ApoA-IM used for rice transformation. b) Restriction analysis of expression vector to confirm the correct insertion of gene and promoter; M molecular weight marker; 1 SpHI restriction analysis (linearized plasmid and 724bp, and 1990 bp fragments); 2 EcoRV restriction analysis (linearized plasmid and 2624 bp, 2177 bp, and 1135 bp fragments); 3 XbaI/EcorI restriction analysis (linearized plasmid and 1015 bp, and 730 bp fragments); 4 EcoRI restriction analysis (linearized plasmid and 1745 bp fragment); 5 SacI/HindIII restriction analysis (linearized plasmid and 740 bp, and 360 bp fragments). The restriction analysis confirms the correct insertion of ApoA-IM gene and 13 KDa prolamin promoter.

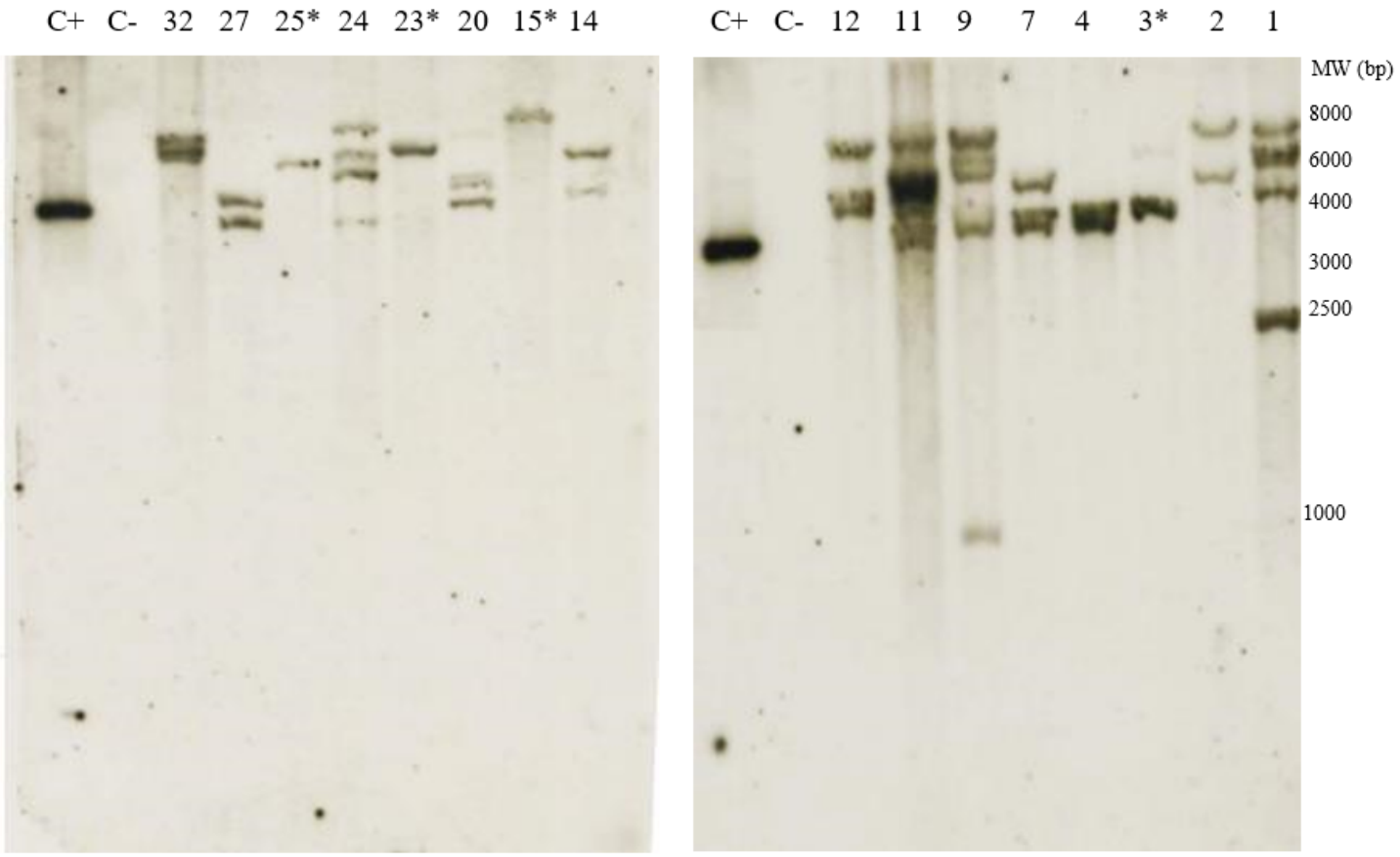

Figure 2

Southern Blot analysis of transgenic rice plants. DNA was digested with XbaI, separated in a 0,8% agarose gel, transferred to a nylon membrane, and probed with a DIG-labeled APOA-IM gene. C+ positive control (3732 bp XbaI fragment from pGEMT-ApoA-IM); C- untransformed control plant;1,2,3,4,7,9,11,12,14,15,20,23,24,25,27, and 32 ID of transgenic rice plants. The transgenic rice plants that present ApoA-M in a single copy was marked with *

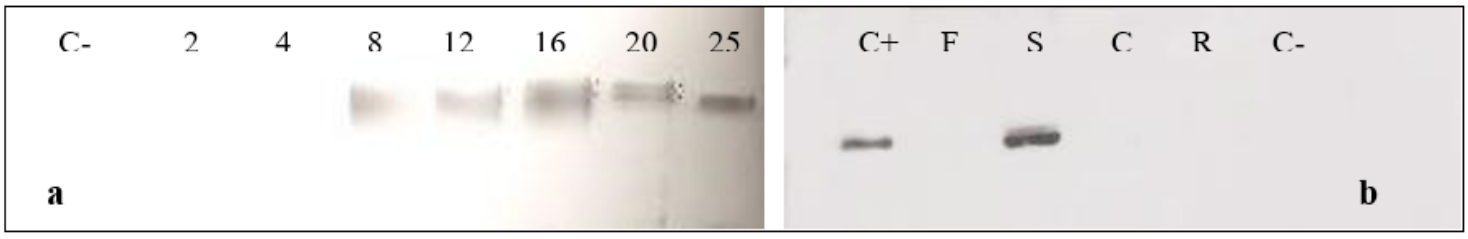

Figure 3

a) Western blotting in reducing conditions (with β-mercaptoethanol). Rice seeds of the same panicle collected on different days after flowering (2,4, 8, 12, 16, 20, and 25 DAF); C- rice seeds of an untransformed plant. b) Western blotting in reducing conditions performed on different plant tissues of the same rice plant transformed. C+ recombinant hApoA-I; F proteins extracted from the leaf; S proteins extracted from transformed rice seeds; C proteins extracted from culm; R proteins extracted from roots. C-

rice seeds untransformed. The presence of the signal corresponding to positive control confirms the seed-specificity of 13 KDa promoter.

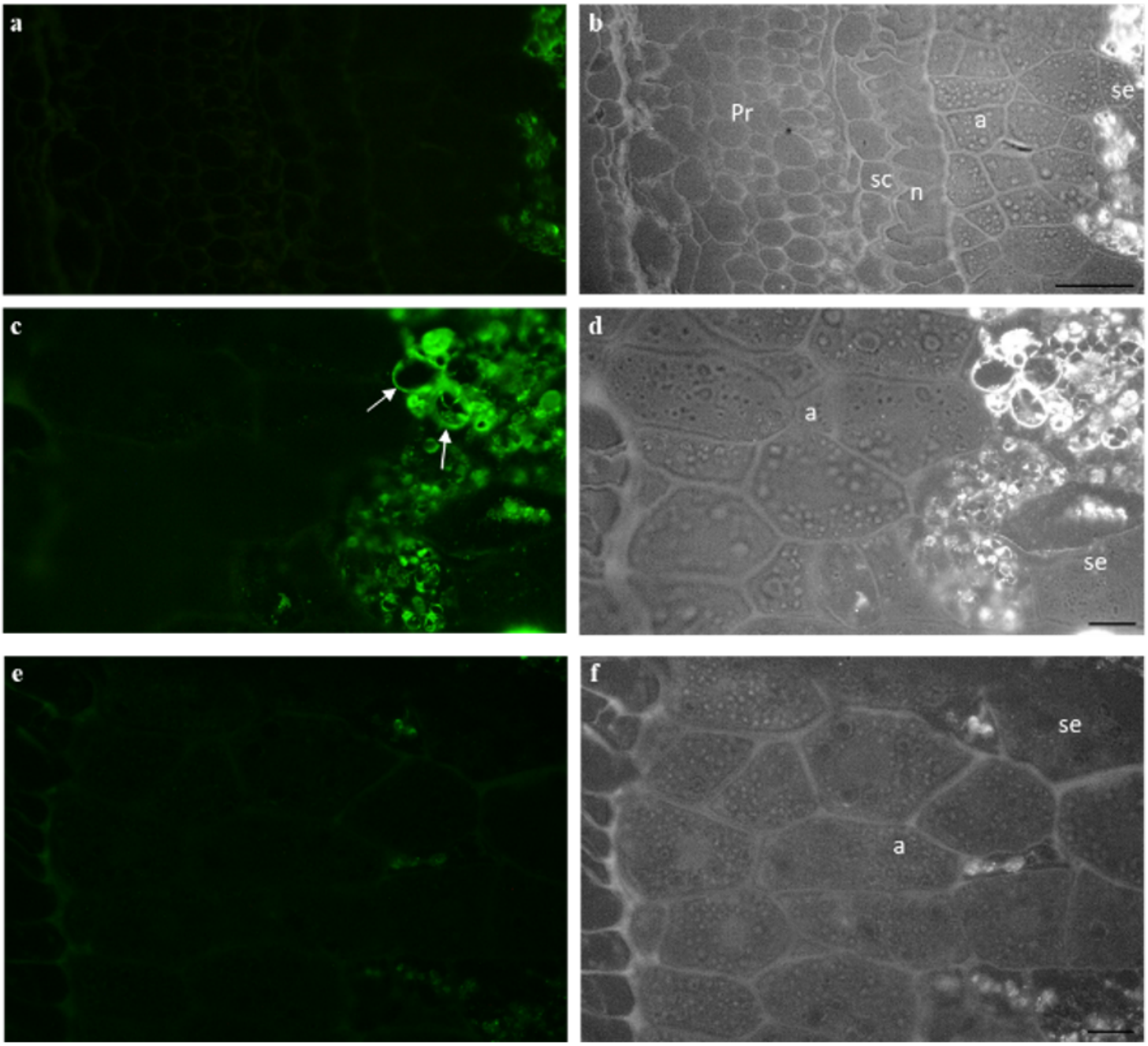

Figure 4

a-d Immunofluorescence (a,c) and bright field images (b,d) of caryopsis I of ApoAI M transformed plant. Pericarp, seed coat, nucellus, aleurons cells, and endosperm cells were observed (Pr, Sc, N, a, and se respectively). A high fluorescence was observed only in the amyloplasts of endosperm cells particularly in the stroma, surrounding starch granules (a.c, arrows). e, f: Negative controls showed no cross-reaction with seed tissues. Magnification bar: b: 20 µm, d, f: 5 µm.

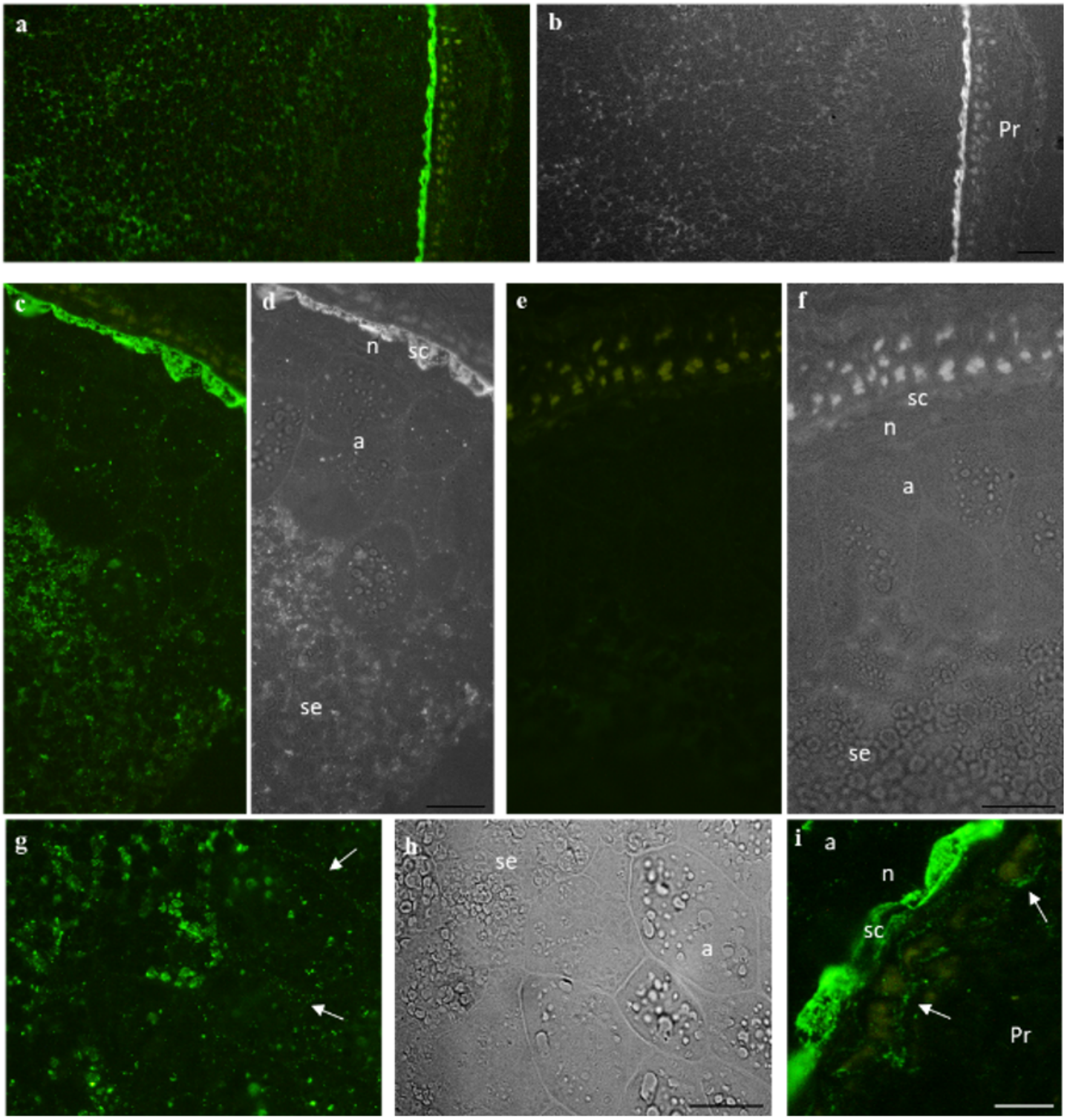

Figure 5

a-i Immunofluorescence (a,c,e,g,i) and bright field images (b,d,f,h) of caryopsis II of ApoAI M transformed plant. Pericarp, seed coat, nucellus, aleurons cells, and endosperm cells were observed (Pr, Sc, N, a, and se respectively). Pericarp and nucellus appeared thinner (a,b and cf respectively). ApoA-IM was localized in organelles in endosperm cells, seed coat, and aleurone cells (c,d; g-h). High fluorescence was observed in the seed coat cell cytoplasm (a, c, i) and ApoA-IM is localized in the cell wall of pericarp cells (i, arrows)

and aleuron cells (g,h; arrows). Only autofluorescence was observed in negative control (e, f). Magnification bar: b: 20 µm, d, f, h, i: 10 µm.

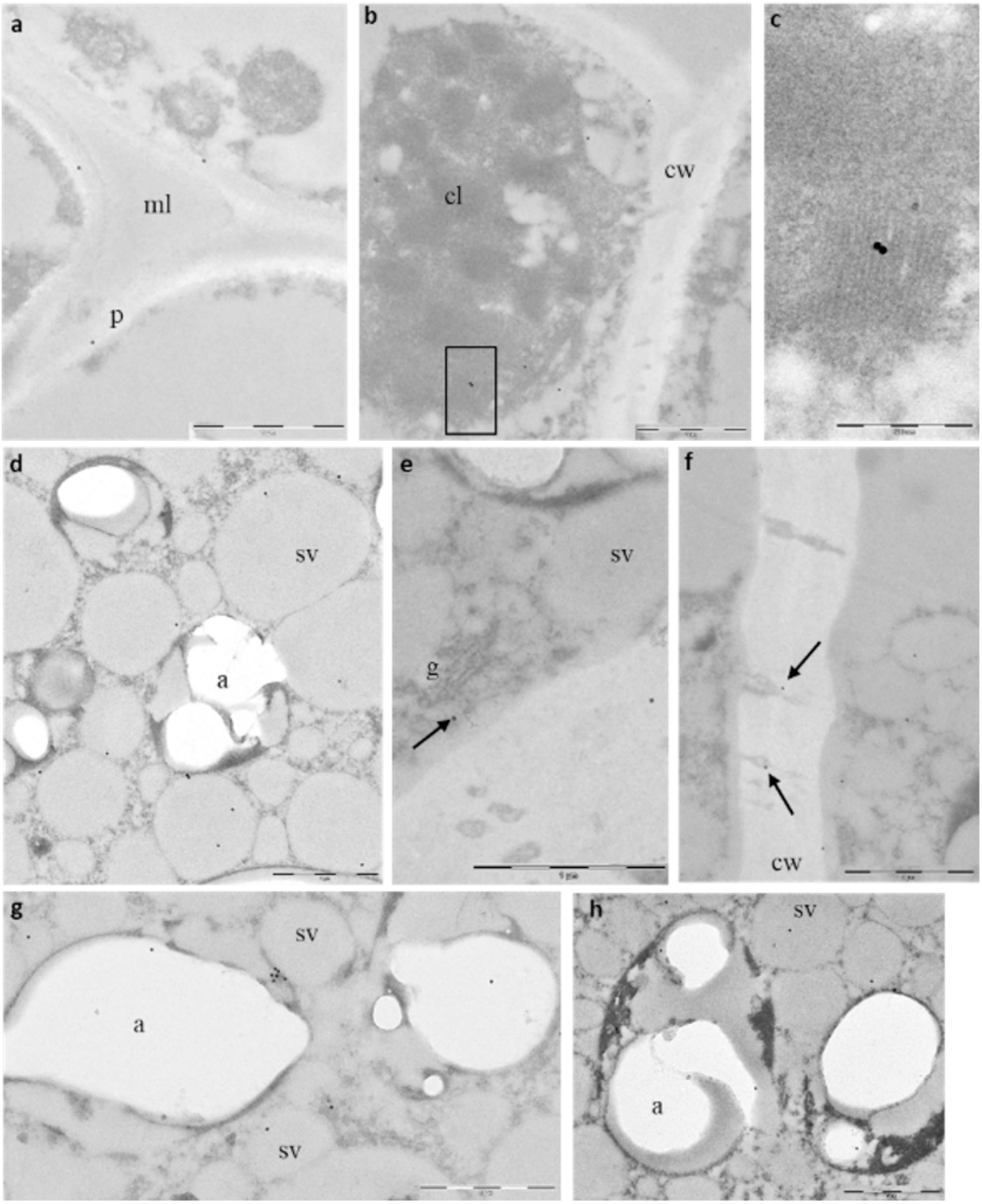

Figure 6

immunogold analyses on caryopsis II of ApoAI M transformed plant. In the seed coat, ApoA-IM was localized in the primary cell wall (a; p: primary cell wall, ml: middle lamella) and at the grana into chloroplasts (b, c; cl: chloroplast, cw: cell wall). In aleuron cells ApoA-IM localised in in the peripheral area

of storage vacuoles (d; sv: storage vacuole, a: amyloplast), in vesicles associated with dictyiosomes (e; arrow; g: Golgi body) and in the cell wall, sometimes into plasmodesmata (f; arrow). In the endosperm, ApoA-IM localized both in storage vacuoles and in amyloplasts and occasionally in the cytoplasm (g,h). Magnification bar: a,b,d-h: 1 µm, c: 500 nm.

Supplementary Files

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