In Vivo Characterization of Human *APOA5* Haplotypes

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ABSTRACT

Increased plasma triglycerides concentrations are an independent risk factor for cardiovascular disease. Numerous studies support a reproducible genetic association between two minor haplotypes in the human apolipoprotein A5 gene (*APOA5*) and increased plasma triglyceride concentrations. We thus sought to investigate the effect of these minor haplotypes (*APOA5*2* and *APOA5*3*) on ApoAV plasma levels through the precise insertion of single-copy intact *APOA5* haplotypes at a targeted location in the mouse genome. While we found no difference in the amount of human plasma ApoAV in mice containing the common *APOA5*1* and minor *APOA5*2* haplotype, the introduction of the single *APOA5*3* defining allele (19W) resulted in 3-fold lower ApoAV plasma levels consistent with existing genetic association studies. These results indicate that S19W polymorphism is likely to be functional and explain the strong association of this variant with plasma triglycerides supporting the value of sensitive *in vivo* assays to define the functional nature of human haplotypes.

Keywords: *APOA5*, triglycerides, cardiovascular disease, haplotypes, genetically engineered mice

INTRODUCTION

Elevated lipid plasma levels are a significant risk factor for stroke and coronary artery disease [1, 2]. The apolipoprotein gene cluster (*APOA5/APOA4/APOC3/APOA1*) on human chromosome 11 (Fig. 1a) is one locus that has been genetically linked to inter-individuals differences in plasma lipid levels [3, 4]. Specifically, *APOA5* is one member of this cluster that has been strongly implicated in influencing plasma triglyceride levels in both mice and humans [5]. Mice lacking *apoa5* have increased plasma concentrations, while mice over-expressing human *APOA5* exhibit decreased plasma triglyceride concentrations [5]. In humans, numerous studies in several different ethnic populations have shown significant association between two minor *APOA5* haplotypes, *APOA5*2* and *APOA5*3*, and elevated plasma triglyceride levels [5-12]. It is estimated that 53% of Hispanics, 35% of African-Americans, and 24% of Caucasians carry at least one of these two minor haplotypes [6], thus suggesting these haplotypes may be a common risk factor for atherosclerosis in humans.

Recent *in vitro* studies have provided initial clues to the possible functional alleles within these minor *APOA5* haplotypes. In humans, genetic studies showed that carriers of the minor *APOA5*2* haplotype [defined by the following nucleotide changes -1131T>C (rs662799), -3A>G (rs651821), 751G>T (rs2072560), and 1891T>C (rs2266788)] displayed significantly lower ApoAV levels compared to the common -1131T variant (152.4 ng/ml versus 200.8 ng/ml) as well as elevated triglyceride levels (1.46 mmol/l versus 0.88 mmol/l)[13]. However, subsequent *in vitro* functional studies of -1131C and

other *APOA5*2* variants yielded no significant alteration in transcription or translation in cell lines [14]. One possible explanation for this observation is based on the strong linkage disequilibrium of the *APOA5*2* haplotype with three neighboring *APOC3* variants [15] (-482C>T, -455T>C, and 3238G>C) that have been repeatedly associated with elevated plasma triglyceride concentrations in several human populations [7, 16-30]. Based on all these results, it remains unclear whether the *APOA5*2* haplotype contributes to elevated plasma triglycerides through a cooperative effect of all four *APOA5* variants or in association with linked causal *APOC3* variants.

In contrast, the *APOA5*3* haplotype associated with elevated triglyceride concentrations is defined by a single coding variant allele [c56G (rs3135506), changing 19S to 19W] and *in vitro* studies revealed a functional consequence of this resulting amino acid substitution [14]. Specifically, molecular modeling of the W19 variant predicted reduced translocation across the endoplasmic reticulum, which was functionally supported through *in vitro* studies indicating a two-fold reduction in protein secretion in comparison to the common 19S allele at this position [14]. In addition, unlike *APOA5*2*, *APOA5*3* showed no association with any neighboring *APOC3* SNP allele [15], suggesting that it is an isolated genetic variant that is functionally responsible for the *APOA5*3* haplotype association with elevated triglyceride levels.

To further assess whether minor human *APOA5* haplotypes by themselves result in altered ApoAV plasma levels *in vivo,* we inserted *APOA5* haplotype constructs into mice through targeted embryonic stem (ES) cell engineering. To circumvent the standard

mouse transgenic complications of variation in copy number and integration sites, we inserted each haplotype construct into a vector designed to rescue an ES cell harboring a partially defective hypoxanthine phosphoribosyltransferase gene (*hprt*), thereby allowing for appropriately targeted colonies to be positively selected using Hypoxanthine Aminopterin Thymidine (HAT) medium [31]. Through these studies, we find that while *APOA5*2* failed to result in any considerable change in ApoAV plasma levels compared to *APOA5*1*, introduction of the W19 variant into the minor *APOA5*2* haplotype significantly reduced ApoAV plasma levels *in vivo*.

RESULTS

Generation of APOA5 Haplotype Mouse Lines

To generate each *APOA5* haplotype construct, we electronically identified 2 publicly available bacterial artificial chromosome (BAC) clones that corresponded to haplotypes *APOA5*1* (BAC CTC-270C21) and *APOA5*2* (BAC RP11-442E11). From these BACs, we cloned an 11,360 bp fragment (chr11:116,163,727-116,175,086; hg18) that corresponds to the human *APOA5* region (Fig. 1b; see Methods). The minor *APOA5*3* haplotype was then constructed through site-directed mutagenesis of the cloned *APOA5*1* haplotype, creating a single change of c56C to c56G (a.k.a. S19 to 19W). In addition, to ascertain the consequence of the 19W change we generated a fourth hybrid haplotype of *APOA5*2* and *APOA5*3* through similar site-directed mutagenesis of cloned haplotype *APOA5*2*, changing c56C to c56G, hereon referred to as *APOA*4* (Fig. 1c).

All four haplotypes were cloned into an *hprt* replacement vector [31] and each construct was independently electroporated into a previously described E14TG2a *hprt*-deficient 129 ES cell line (Fig. 1d) (see methods) [32]. Colonies resistant to HAT medium were screened using PCR and Southern analysis (data not shown) and were subsequently injected into C57BL/6J blastocyst stage embryos. The resulting male chimeras were bred to C57BL/6J females to allow for detection of ES cell transmission based on the 129 strain cell line derived agouti coat color marker. Female agouti mice positive for the

transgene were bred to C57BL/6J males to generate transgene positive hemizygous males (*hprt* is located on the X-chromosome). While haplotypes *APOA5*1*, *APOA5*2*, and *APOA5*4* were successfully transmitted through the germline, none of the resulting chimeric mice from two separate *APOA5*3* ES clones did so, despite repeated attempts. We thus limited our analysis to aforementioned mouse lines.

*Plasma ApoAV levels are reduced for Haplotype APOA5*4 but not APOA5*2 Mouse Lines*

To date, *APOA5* expression has only been detected in the liver where it is thought to be secreted into the plasma, functioning in increasing lipolysis in the periphery as well as LDL clearance by the liver. We thus sought to determine if ApoAV plasma levels were altered due to the allelic variants found within their respective haplotypes. We measured human ApoAV plasma levels in male mice using a human-specific ApoAV antibody. As a control, we found no detectable levels of human ApoAV in littermates that were negative for the transgene (n=18). In contrast, we detected an average ApoAV plasma level of 0.628 mg/dL in transgenic mice for the most common human *APOA5*1* haplotype (Fig. 2a). It is worth noting that these levels are considerably lower than the average human levels that are estimated to be 17.92 ± 7.48 mg/dL [13]. We next determined if the minor *APOA5* haplotypes associated with increased triglyceride levels in humans displayed altered ApoAV plasma levels compared to *APOA5**1. No significant difference (0.628 versus 0.593mg/dL; p=0.810, t-test) was observed between *APOA5*1* and *APOA5*2* transgenic mice. In contrast, *APOA5*4* which contains a single

S19 to W19 change within *APOA5*2*, showed significantly lower ApoAV plasma levels compared to *APOA5*1* (0.173 versus 0.628 mg/dL; p=0.002, t-test) (Fig. 2a). Combined, these results indicate that while the *APOA5*2* variants do not have a significant effect on ApoAV plasma levels, the W19 allele alone resulted in substantially lower ApoAV levels.

Finally, we measured triglyceride levels in mice derived from all *APOA5* haplotype lines observing no differences between these cohorts (Fig. 2b). This was not unexpected since the endogenous mouse locus for *apoa5* remains intact within these *hprt*-based targeted mice. Future studies aimed at determining the effects of these transgenes with *apoa5*-deficient mice would directly assess the ability of these haplotypes to recapitulate the triglyceride phenotypic difference found in humans. Nevertheless, the established role of decreased ApoAV function leading to increased triglycerides in knockout mice, is consistent with human *APOA5* minor haplotypes leading to lower ApoAV protein levels which in turn raise triglycerides in plasma.

DISCUSSION

Our goal in this study was to determine the *in vivo* effect of *APOA5* polymorphisms repeatedly associated with inter-individual differences in plasma triglyceride concentrations. Using an *hprt* mouse targeting scheme, we created male mice each carrying a different single copy of a human *APOA5* haplotype. We were thus able to avoid the complexities of regular transgenic mice where multiple copies and different insertion sites can significantly effect the expression of a transgene. In addition, this transgenic scheme enabled the disconnection of *APOA5* from the neighboring *APOC3* (as well as *APOA4* and *APOA1*), consequently revealing that only one of the two minor haplotypes associated with increase triglycerides in humans appear causative of this plasma phenotypic difference across humans.

Previous *in vitro* functional studies on the majority of variants defining *APOA5*2* have suggested that the variants within this haplotype are not functional [14]. However, these studies were conducted within cell lines and were not able to assess all haplotype defining alleles in combination; but rather studied each independently based on predictions on how they may alter gene function. By inserting the entire *APOA5*2* haplotype into the mouse, we find that these alleles combined do not have a cooperative effect that might otherwise support their causal role to the observed human genetic association with increased triglycerides. These findings further suggest this *APOA5*2* haplotype association may be explained due to its strong linkage disequilibrium with previously characterized triglyceride elevating *APOC3* alleles. It will be interesting in

future studies to determine the effect of *APOC3* minor haplotypes associated with increase triglycerides through similar *in vivo* studies.

We can strongly infer that the S19W variant is functional. This is based on the observation that the single change of *APOA5*2* haplotype S19 to W19 resulted in a three-fold decrease in ApoAV plasma levels *in vivo*. This decrease is consistent with previous functional studies that correlate this change with a significant reduction in ApoAV secretion levels *in vitro* [14]. The phenotypic outcome of such a reduction, elevated plasma triglyceride levels, can be inferred from *apoa5* mouse knockout mice. *Apoa5* null homozygous mice have four times as much plasma triglyceride levels as their wild-type littermates[5], thus indicating that the expected phenotype of lower ApoAV would be higher plasma triglyceride levels. In conclusion, these results further support individuals carrying the *APOA5*3* haplotype have a higher risk for elevated plasma triglyceride levels directly through the S19W genetic variant that defines this haplotype.

While this study was focused on *APOA5* specific haplotypes, the results are relevant to the increasing number of genetic associations being identified in humans. These statistical associations gain power through validation in independent cohorts, but ultimately lack formal proof of their causative nature. Through a combination of *in vitro* and *in vivo* studies as described here it is anticipated that haplotypes can be functionally linked to their associated human phenotypes and provide an experimental means to define the ultimate functional allele(s) within these stretches of human DNA.

MATERIALS AND METHODS

Cloning the APOA5 hprt constructs

BACs 270C21 (*APOA*1*; GenBank AP001481) and 442e11 (*APOA*2*; GenBank AC007707) were restriction digested sequentially with *Not* I and *BstE* II and separated on a pulse field gel without ethidium bromide. Electronically predicted 11.5 kb fragments were gel excised and purified using QiaEx II kit (Qiagen, Valencia, CA), blunt-end repaired using EpiCenter CopyControl PCR Cloning kit (Epicentre Madison, WI), and ligated into a pCCI vector (Epicentre Madison, WI). 5kb *Hind* III fragments from both the *APOA5*1* and *APOA5*2* clones in pCCI were cloned into a pBS vector (Invitrogen) to facilitate the ease of site-directed mutagenesis. In both vectors site-directed mutagenesis of the *APOA5*3* variant (c56C>G) was carried out using Quickchange site-directed mutagenesis kit (Stratgene}. After mutagenesis clones was re-verified by sequencing with primers F1-AGAGGCCTCAGCTTTTCCAGGA, R1-GCGGATCCGAGCAGAGCAGA, R2-GCGGATCCGAGCAGAGCAGG, R3-TCTGGCTCTTCTTTCAGCGTTTTC, and R4-TCTGGCTCTTCTTTCAGCGTTTTG. Next, 5kb *Hind* III fragments were gel excised from pBS and ligated back into the pCCI thus generating haplotypes *APOA5*3* and *APOA5*4*. We then cloned these sequences into the pMP8NEB delta lacZ vector[31] (a kind gift from Sarah K. Bronson) using *Not* I. To verify there were no mistakes in orientation and nucleotide sequence of *APOA5* and the haplotype determining alleles, we analyzed these vectors using restriction enzyme based analysis and sequencing.

hprt targeting

All 4 *APOA5* haplotypes within the pMP8NEB delta lacZ vectors were linearized with *Not* I restriction enzyme and electroporated into a E14TG2a hprt-deficient 129 ES cell line[32]. Cells were grown on murine embryonic fibroblast in in DMEM (Gibco) supplemented with 10% fetal bovine serum (Hyclone), L-Glutamine (Gibco), non-essential amino acids (Gibco), HEPES (Gibco) Beta-mercaptoethanol (Sigma), penicillin/streptomycin (Gibco) and ESGRO (Chemicon). Positive colonies were selected for using HAT medium and were screened by PCR using primers that spanned the mouse *hprt* gene and human AV gene junction. Sequence for primers are: mHPRT F 5'TGAGTTCCTGCATTGAGCAACTGA3' and hApoAV R 5'TGAGATGCAGAGGGGACACTTGG 3'. For Southern blot 10 μg of genomic DNA was digested with *Sca* I and the probe was synthesized using the following PCR primer pair: 5' TTATGGTACTGGCAGGGAGATTAGG3' and 5'CAGAACATCTTGAAACCCAGCATCC 3'. The resultant male chimeras were bred to C57BL/6J females, and heterozygous females generated from these crosses were mated with C57BL/6J males to generate transgene positive hemizygous males.

Triglyceride measurements

All animals were fed standard chow diet and were analyzed between 2 to 4 months of age. Blood samples were collected after a 5-hour fast by retro-orbital bleeding using

heparinized microhematocrit tubes. Total triglyceride concentrations were measured using enzymatic methods (Sigma 337-A) in 96 well plates using a Spectramax 250 (Molecular Devices).

APOA5 measurements

A pool of two monoclonal anti-human apoAV antibodies, raised in mice by using recombinant protein, was used at 10 μg/ml in PBS 0.1M pH 7.2 to coat the wells of the microtiter plates at room temperature overnight. The wells were washed twice with PBS 0.1M and then saturated with 3% BSA/PBS for 1h at 37°C. For quantitation, a pool of human plasma was calibrated and titrated using apoAV recombinant protein as a primary standard. Then, the pool of plasma was used for the calibration curve. All dilutions were done in the blocking buffer (1% BSA/PBS). One hundred microliters of the antigen solution was added to the wells and incubated for 2h at room temperature. The wells were washed four times with PBS followed by an addition of an anti-apoAV polyclonal antibody (1:400), produced in goat using synthetic peptide, and incubated for 2h at 37°C. Unbound antibody was removed by washing four times, and 100 μl of goat anti-rabbit IgG-biotin conjugate diluted (1:20000; Sigma) was added to each well. The plates were incubated for 1h at 37°C and washed several times. Following incubation with the biotinylated antibody, a solution of streptavidin-alkaline phosphatase (1:500; Sigma) was added for 30 min at 37°C. Then, detection using p-nitropheny phosphate substrate (pNPP; Sigma) was performed in the dark at room temperature and the absorbance at 405 nm was measured using a microplate photometer (Dynex Technologies).

ACKNOWLEDGEMENTS

We thank Veena Afzal and Malak Shoukry for technical help. Research was conducted at the E.O. Lawrence Berkeley National Laboratory, supported by Grant HL071954 and performed under Department of Energy Contract DE-AC02-05CH11231, University of California.

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FIGURE LEGENDS

Figure 1: Generation of *APOA5* haplotype mouse lines. A) Apolipoprotein gene cluster on human chromosome 11. B) *APOA5* gene and location of haplotype determining variants. C) *APOA5* haplotype constructs D) Recombination of the *APOA5* haplotype constructs into a defective *hprt* locus in the mouse.

Figure 2: ApoAV and triglyceride plasma levels in *APOA5* haplotype mice. A) ApoAV plasma levels. B) Triglyceride plasma levels. Error bars indicate the standard error, 'N' below the chart indicates the number of mice assayed, followed by the mean ApoAV or triglyceride plasma levels (mg/dL) and the standard error in each experiment.

FIGURE 1

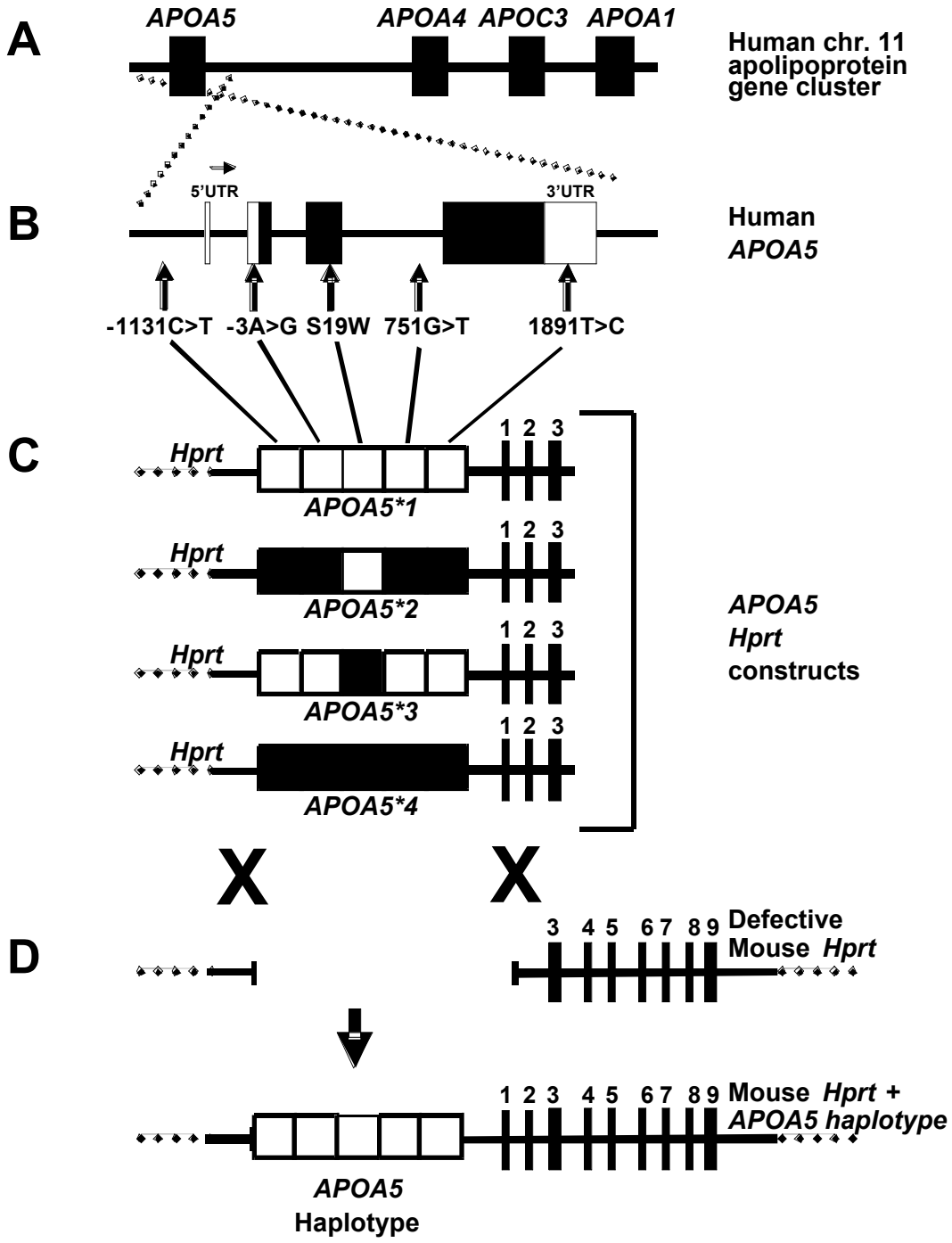
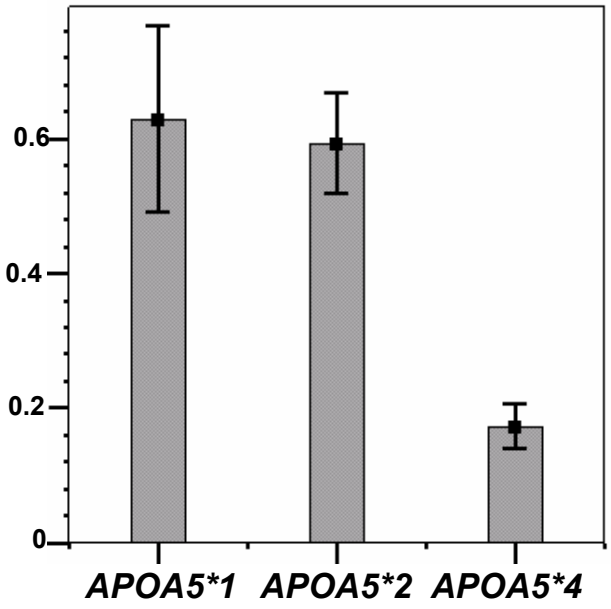

A
APOA5
APOA4
APOC3
APOA1
Human chr. 11 apolipoprotein gene cluster
B
5'UTR
3'UTR
Human APOA5
-1131C>T
-3A>G
S19W
751G>T
1891T>C
C
Hprt
APOA5*1
APOA5*2
APOA5*3
APOA5*4
1 2 3
APOA5 Hprt constructs
X
X
D
3 4 5 6 7 8 9
Defective Mouse Hprt
1 2 3 4 5 6 7 8 9
Mouse Hprt + APOA5 haplotype
APOA5 Haplotype

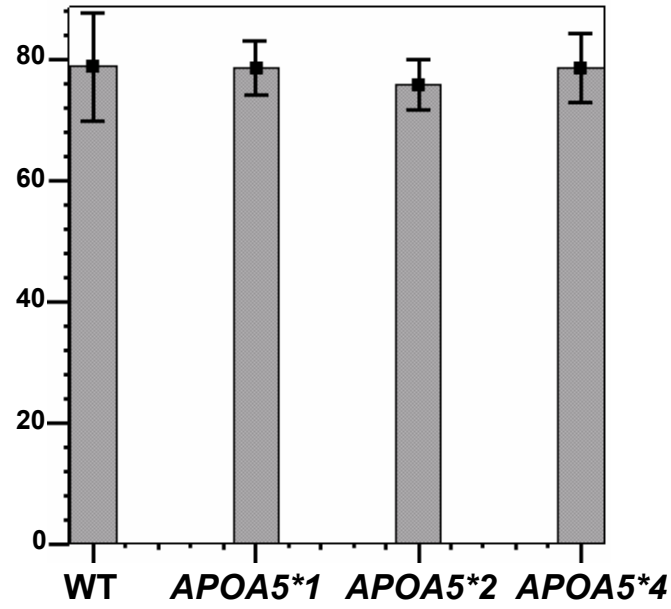
FIGURE 2

A



N	18	43	20
Mean	0.628	0.593	0.173
Std. error	0.138	0.075	0.033

B



N	6	12	7	6
Mean	78.98	78.67	75.95	78.75
Std. error	8.97	4.39	4.31	5.66