Arabidopsis thaliana extracts optimized for polyphenols production as potential therapeutics for the APOE-modulated neuroinflammation characteristic of Alzheimer’s disease in vitro

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Although the cause of Alzheimer’s disease (AD) is unknown, glial-induced neuroinflammation is an early symptom. Familial AD is caused by increases in amyloid-beta (Aβ) peptide, particularly soluble oligomeric (oAβ), considered a proximal neurotoxin and neuroinflammatory stimuli. APOE4, a naturally occurring genotype of APOE, is the greatest genetic risk factor for AD; increasing risk up to 12-fold compared to APOE3 and APOE2. oAβ-induced neuroinflammation is greater with APOE4 compared to APOE3 and APOE2. As sinapates and flavonoids have anti-inflammatory properties, a protocol was developed for optimizing polyphenol production in seedlings of Arabidopsis thaliana (A. thaliana). Three mutants (cop1, prn1, xpf3) were identified, and the extracts treated with liver microsomes to mimic physiological metabolism, with HPLC and MS performed on the resulting metabolites for peak identification. These extracts were used to treat primary glial cells isolated from human APOE-targeted-replacement (APOE-TR) and APOE-knock-out (KO) mice, with neuroinflammation induced by lipopolysaccharide (LPS) or oAβ. The dose-response data for TNFα secretion demonstrate the followed order: APOE-KO > APOE4 > APOE3 > APOE2, with xpf3 the most effective anti-neuroinflammatory across APOE genotypes. Thus, the plant-based approach described herein may be particularly valuable in treating the APOE4-induced neuroinflammatory component of AD risk.

Accumulating evidence supports the anti-oxidant and anti-inflammatory effects of polyphenols, including phenylpropanoids and its sub-classes of flavonoids, sinapate esters, and hydroxycinnamic acids, suggesting a potential role in a therapeutic approach to human inflammation-related diseases1-3, including neurodegeneration4-5. While dietary flavonoids have been shown to possess antioxidant, anti-inflammatory, and neuroprotective effects6-7, isolates of single compounds have limited success8-9. For example, both in vitro and in vivo studies of the polyphenol resveratrol indicate that half-life, bioavailability and solubility issues present major obstacles to its utility (reviewed in ref. 10). Arabidopsis thaliana (A. thaliana) is a useful model for the development of mutants optimized to produce polyphenols. Polyphenol-enriched extracts derived from mutants offer several advantages for the study of potential medicinal qualities of plant extracts: (1) the genome is mapped and sequenced; (2) many chemical constituents are known; (3) the mutants can be grown at a low cost, and (4) seedling growth requires

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only a small amount of time and space. Thus, polyphenol-enriched extracts can be easily and quickly obtained. Indeed, we recently demonstrated that specific flavonoids preferentially accumulate in the prn1 (gene At3g59220) A. thaliana mutant11.

Alzheimer’s disease (AD) is the most common neurodegenerative disease of aging. Its pathological hallmarks include extracellular amyloid plaques, composed of amyloid-β (Aβ) peptide, and intracellular neurofibrillary tangles composed of abnormally phosphorylated tau protein, as well as neuron loss14. While amyloid plaque density may not correlate with AD-specific dementia, oligomeric Aβ (oAβ), a soluble aggregate of the peptide, has been identified as a proximal neurotoxin in human AD patients and familial AD-transgenic (FAD-Tg) mouse brains, as well as in vitro (reviewed in ref. 15). Importantly, in vitro studies demonstrate that oAβ elicits a neuroinflammatory response16–18. In AD, glial activation may be Aβ-independent, i.e. via damaged cells, or Aβ-dependent via increased soluble oAβ or amyloid plaques. Neuroinflammation due to chronically activated glia may both initiate and further exacerbate the pathogenesis of AD (for review)19–21. This creates a neuroinflammatory phenotype that is likely a driving force for early cognitive impairment20–27. Polyphenol extracts including curcumin, resveratrol and gallic acid derivatives reduce cytokine and chemokine responses in some AD models, although the specific impact on neuroinflammation is still poorly understood28–30. In addition, studies have demonstrated that dietary flavonoids preserve cognitive function during aging, and reduce the risk for AD and dementia7–9. The high consumption of polyphenolic-rich vegetables, fruit juices, and red wine has been shown to delay the onset of AD and dementia6–14. In FAD-Tg mouse models, administration of the green tea flavonoid EGCG reduced Aβ pathology with an improvement in cognition54,55, while blueberry extracts prevented deficits in cognition without altering the Aβ burden35. In vitro, flavonoids have been shown to reduce glial-mediated inflammation46–51.

The ε4 allele of the APOE gene is the greatest genetic risk factor for AD12,25. Risk is increased approximately 5–12-fold for carriers of one or two copies of the APOE4 allele compared to APOE3, whereas APOE2 reduces risk 2–4-fold44. In humans, apolipoprotein E (ApoE) has three isoforms that differ by a single amino acid substitution at residues 112 or 158: apoE2 (Cys 112,158), apoE3 (Cys112Arg158) and apoE4 (Arg12,158). ApoE is the only apolipoprotein expressed within the central nervous system (CNS) and apoE-containing lipoproteins are produced primarily by astrocytes. While the impact of apoE on brain function is multifactorial, both in vivo55 and in vitro18 data demonstrate that apoE4 is associated with a greater neuroinflammatory response than apoE356. While multiple lines of evidence demonstrate a heightened neuroinflammatory response with APOE4 compared to APOE3, particularly in response to lipopolysaccharide (LPS; endotoxin), a ligand for the toll-like receptor 4 (TLR4) surprisingly little data address APOE-modulation of Aβ-induced neuroinflammation18. In addition, APOE4 carriers can respond anomalously in clinical trials, sometimes negatively27–30. While epidemiological data indicate that non-steroidal anti-inflammatory drugs (NSAIDs) preferentially lower AD risk in APOE4 carriers11,32, results from anti-inflammatory clinical trials indicate that drug efficacy will be highly context dependent (reviewed in ref. 63). For example, the conclusion from the AD Anti-inflammatory Prevention Trial (ADAPT) was that neither naproxen nor celecoxib prevented AD64–72. However, confounding factors include the short treatment duration, low AD incidence rate, and, importantly, the age of enrollment (≥70).

Thus, new therapeutic strategies targeting the neuroinflammation characteristic of AD, particularly for APOE4 carriers, are critical. A. thaliana mutants may provide a novel approach for producing extracts enriched in flavonoids and polyphenols. Three such mutants were used to treat primary glial cells isolated from human APOE-targeted-replacement (APOE-TR) and APOE-knock-out (KO) mice, with neuroinflammation induced by LPS or oAβ. As a measure of microglial activation, tumor necrosis factor (TNF)-α, secretion followed the order: APOE-KO > APOE4 > APOE3 > APOE2, with xpβ mutant the most effective anti-neuroinflammatory across genotypes. Thus, the plant-based approach described herein may be of economic importance to produce an effective and viable method to target neuroinflammation, one of the earliest symptoms of AD, in a form of a nutraceutical or dietary supplement.

**Results**

**UV-C survival screen for A. thaliana mutant seedlings with increased polyphenol production.** We screened 6 day (d)-old seedlings of specific mutants of A. thaliana and identified 3 mutants that survived UV-B irradiation comparable to wild type (wt) (Fig. 1A, top) and survived UV-C irradiation significantly better than wt and the adt3 mutant, a mutant designed for reduced phenylalanine levels, thus the negative control (Fig. 1A, bottom). These responses are indicative of effective polyphenol accumulation or activity41,52. The mutants are: 1. Pirin1 (transcriptional co-factor and quercetinase; prn1); 2. constitutive photomorphogenenic1 (E3 ubiquitin-protein ligase; acts as a repressor of photomorphogenesis; cop1); and 3. excision repair homologue 3 (homolog of the human xeroderma pigmentosum group F DNA repair; xp3).

The CellRox assay was used to assess oxidative stress, an additional measure of enhanced polyphenols in the mutants, as polyphenols can be potent scavengers of reactive oxygen species (ROS). Post UV-C irradiation, epidermal optical slices (1 μm thick) were stained for the CellRox assay with fluorescence by Cy5 indicating ROS levels (670 nm, pink), while DAPI (358 nm; blue) and FITC (448 nm; green) capture the natural fluorescence of phenylpropanoids33. Representative images were captured on a deconvoluting microscopy for wt, wt + UV-C and xpβ + UV-C (Fig. 1B, top). Quantification of fluorescence for Cy5 (650 nm, pink, ROS) and DAPI + FITC (359–494 nm; green-blue, polyphenol levels) are represented as bar graphs (Fig. 1B, bottom). With UV treatment, the xpβ mutant had significantly reduced signs of oxidative stress compared to wt. The xpβ mutant seedlings also produced significantly more phenylpropanoids than wt.

Using principal components analysis (PCA), independent batches of seedling extracts clustered based on mutations (Fig. 2). For PCA of the mutants xpβ, prn1, adt3, cop1 and wt samples, using relative content of peaks from UV spectroscopy (under UV 254 nm UV 280 nm and UV 320 nm) as variables, demonstrates that the samples formed their own cluster within a small region significantly seperated from each other. The exception was the similarity of
Identification of extractable constituents of *A. thaliana* mutant *xpf3* extract, pre- and post-treatment with liver microsomes. HPLC revealed 13 identifiable peaks from 10 samples of each mutant extract. A sample trace for *xpf3* extract (Fig. 3) pre- (lower) and post-(upper) treatment by murine liver microsomes (LM) used in order to mimic aspects of metabolism. LM treatment induced a quantitative increase in most of the 13 identified constituents, particularly peak 7, barely visible in the untreated extract (lower trace). The contents of the peaks were analyzed by HPLC-DAD-MS/MS (described in Methods), and structures confirmed by NMR (Table 1).

Plant extracts dose-dependently attenuate APOE-modulated LPS-induced neuroinflammation (*APOE-KO > APOE4 > APOE3 ≥ APOE2*). LM-treated mutant extracts were used to treat the primary mixed glial cultures from APOE-TR or APOE-KO mice consisting of ~95% astrocytes + 5% microglia, the approximate proportion found in both human and mouse brain. In this human apoE-relevant *in vitro* model, we measured the amount of TNFα released into the media, based on our previous work demonstrating that the levels

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**Figure 1.** UV-C survival screen for *A. thaliana* mutant seedlings with increased polyphenol production. (a) 6d-old seedlings of specific *A. thaliana* mutants quantified 24 h after sub-lethal (3 min) UV-B (top) or lethal (8 min) UV-C radiation (bottom) demonstrate that mutants *cop1*, *prn1* and *xpf3* survive a dose of UV-C that is lethal to wt and the *adt3* negative control. The number of surviving seedlings for the mutants and wt, for a sub-lethal dose (top) and a lethal dose (bottom); n = 3, expressed as means ± SEM, *vs. wt, p < 0.05 via paired student's t-test (two-tailed) using GraphPad Prism 5. (b) Post UV-C irradiation, epidermal optical slices (1 μm thick) were stained for the CellRox assay with fluorescence by Cy5 indicating ROS levels (670 nm, pink, ), while DAPI (358 nm; blue) and FITC (448 nm; green) capture the natural fluorescence of phenylpropanoids. Representative images were captured on a deconvoluting microscopy for wt, wt + UV-C and *xpf3* + UV-C (Fig. 1B, top). Quantification of fluorescence for Cy5 (670 nm, pink, ROS) and DAPI + FITC (359–494 nm; green-blue, polyphenol levels) are represented as bar graphs (Fig. 1B, bottom). With UV treatment, the *xpf3* mutant had significantly reduced signs of oxidative stress compared to wt. The *xpf3* mutant seedlings also produced significantly more phenylpropanoids than wt. Data expressed as means ± SEM, for n = 20, *vs. wt and wt + UV-C, p < 0.05 via paired student's t-test (two-tailed) using GraphPad Prism 5.
Thus, the data in this figure is based on duplicates for a full dose-response experiment with LPS treated with 4 and was effective in attenuating LPS-induced TNF-α secretion was modulated by α-induced TNF-α levels in the media, were, over-all, significantly higher in APOE4 extracts at the highest dose, the 1:100 dilution. TNF-α levels in the media were measured in duplicate by ELISA, and expressed as %APOE3 LPS-vehicle control (VC) (Fig. 5). The inset is an enlarged graph that more clearly shows the separation between APOE3 and APOE2. The LPS-induced inflammatory response is dose dependent and APOE-modulated (APOE-KO > APOE4 > APOE3 ≥ APOE2) with the greatest change in APOE-KO over the dilution range. At all dilutions, xpf3 was effective in attenuating LPS-induced TNF-α secretion and, as expected, adt3, was the least effective extract. The data, in this figure is based on duplicates for a full dose-response experiment with LPS treated with 4 mutant + wt plant extracts in cultures from 4 APOE genotypes.

**APOE-genotype modulates LPS-induced neuroinflammation with xpf3-induced TNF-α reduction significant with APOE4.** As described, cultures were pre-treated with LM-digested wt or xpf3 plant extracts at two dilutions (1:100 = 150 μg/ml and 1:1000 = 15 μg/ml), or LPS from *Rhodobacter sphaeroides* (LPS-RS) (10 μg/ml), a natural TLR4 antagonist used as a positive control. TNF-α levels in the media, were, over-all, significantly higher in APOE4 cultures compared to APOE3 and APOE2 cultures (Fig. 6). Within all 3 genotypes, LPS-RS, wt (1:100) and xpf3 (1:100) were lower than the VC. Only in the APOE4 cultures was xpf3 (1:1000) significantly lower than VC and, importantly, xpf3 (1:100) significantly lower than wt (1:100).

**Plant extracts dose-dependently attenuate APOE-modulated oAβ-induced neuroinflammation (APOE-KO > APOE4 > APOE3 ≥ APOE2).** As described, cultures were pre-treated with LM-digested wt, prn1, cop1, xpf3 and adt3 plant extracts (log scale dilution: 1:5000, 1:1000, 1:500, 1:200 and 1:100) prior to oAβ (10 μM) to induce a neuroinflammatory response. TNF-α levels in the media (expressed as %APOE3 oAβ-VC) were measured by ELISA, as shown in Fig. 7. TNF-α secretion was modulated by APOE genotype (APOE-KO > APOE4 > APOE3 > APOE2), with the greatest change in APOE-KO over the dilution range. The inset is an enlarged graph that more clearly shows the separation between APOE3 and APOE2. This Figure, as well as Fig. 5 with LPS as the neuroinflammatory stimuli, illustrates the difference between the neuroinflammatory response by genotype. The response of the APOE genotypes to oAβ, while qualitatively similar to LPS, are approximately ½ when expressed as TNF-α levels (%APOE3 VC) (compare Figs 5–7).

| Peak | Identification | M.F./M.W. | RT (min) | U/Vmax | MS (+) (m/z) | MS (−) (m/z) |
|------|----------------|-----------|---------|--------|-------------|-------------|
| 1    | Isomer of 1-O-sinapoyl-β-D-glucose | C_{12}H_{16}O_{16} | 7.187 | 234,323 | 369 [M-H_2O+H]^+ | − | 385 [M-H]^− |
| 2    | Isomer of 1-O-sinapoyl-β-D-glucose | C_{12}H_{16}O_{16} | 7.588 | 224,327 | 369 [M-H_2O+H]^+ | − | 385 [M-H]^− |
| 3    | 1-O-Sinapoyl-β-glucose | C_{12}H_{16}O_{16} | 8.129 | 237,323 | 369 [M-H_2O+H]^+ | − | 385 [M-H]^− |
| 4    | Sinapoyl choline | C_{12}H_{16}O_{16} | 8.473 | 236,327 | 310 [M]^− | 251,207 | − |
| 5    | Quercetin 3-O-β-glucose-7-O-α-rhamnose | C_{12}H_{16}O_{16} | 10.322 | 255,333 | 611 [M-H]^+ | 303 | 609 [M-H]^− |
| 6    | Quercetin 3,7-di-O-α-rhamnose | C_{12}H_{16}O_{16} | 12.985 | 255,347 | 595 [M-H]^+ | 303 | 593 [M-H]^− |
| 7    | N,N'-Di-sinapoyl spermidine | C_{12}H_{16}O_{16} | 13.763 | 241,233 | 558 [M-H]^+ | 352,264 | − |
| 8    | Sinapoyl malate | C_{12}H_{16}O_{16} | 14.739 | 238,329 | − | − | 339 [M-H]^− |
| 9    | Isomer of 1,2-di-O-sinapoyl-β-glucose | C_{12}H_{16}O_{16} | 20.494 | 239,327 | 369 [M-C_6H_5COO+H]^+ | 207,175 | 591 [M-H]^− |
| 10   | 1,2-Di-O-sinapoyl-β-glucose | C_{12}H_{16}O_{16} | 22.022 | 238,328 | 369 [M-C_6H_5COO+H]^+ | 351,207,175 | 591 [M-H]^− |
| 11   | Isomer of 1,2-di-O-sinapoyl-β-glucose | C_{12}H_{16}O_{16} | 22.563 | 239,328 | 369 [M-C_6H_5COO+H]^+ | 351,207,175 | 591 [M-H]^− |
| 12   | Isomer of 1,2-di-O-sinapoyl-β-glucose | C_{12}H_{16}O_{16} | 25.574 | 240,327 | 369 [M-C_6H_5COO+H]^+ | 351,207,175 | 591 [M-H]^− |
| 13   | Isomer of 1,2-di-O-sinapoyl-β-glucose | C_{12}H_{16}O_{16} | 14.739 | 238,327 | 369 [M-C_6H_5COO+H]^+ | 251,207 | 591 [M-H]^− |

Table 1. Peak identification by HPLC-DAD-MS/MS analysis. *The structures were further confirmed by 1H NMR data. 3N,N'-Di-sinapoyl spermidine or one of its isomers, different in the positions of OCH_3 and OH groups.*
APOE-genotype modulates oAβ-induced neuroinflammation with xpf3-induced TNFα reduction significant with APOE4. As described, cultures were pre-treated with LM-digested wt or xpf3 plant extracts at two dilutions (1:100 ≈ 150 μg/ml and 1:1000 ≈ 15 μg/ml), or LPS-RS (10 μg/ml), and inflammation induced by oAβ (10 μM). Similar to LPS, TNFα levels in the media, were, overall, significantly higher in APOE4 cultures compared to APOE3 and APOE2 cultures (Fig. 8). Within all 3 genotypes, LPS-RS, wt (1:100) and xpf3 (1:100) were lower than the VC. It is interesting that LPS-RS blocked oAβ-induced TNFα secretion in all the APOE cultures as LPS-RS is a TLR4 antagonist, suggesting that oAβ-induced neuroinflammation is mediated by this receptor pathway. Only in the APOE4 cultures were wt (1:1000) and xpf3 (1:1000) significantly lower than the VC and, importantly, xpf3 (1:100) was significantly lower than wt (1:100). As for the dose response graphs (Figs 5 and 7), the response of the APOE genotypes to oAβ, while qualitatively similar to LPS, are approximately ½ when comparing TNFα levels (pg/ml) (compare Figs 6–8).

**Discussion**

The studies described herein were designed to test the potential of plant-based materials for the prevention of chronic inflammation as the *A. thaliana* plant extracts were added to the cultures prior to the treatment with LPS or oAβ. Flavonoids can play critical roles in reducing negative effects of oxidative stress in plant cells, but also act as potential anti-inflammatory agents for human use. *A. thaliana* is a genetic model with an established phenylpropanoid synthesis pathway that can be manipulated, and developed for specific chemical applications. Herein, we identified *A. thaliana* mutants, and extractable compounds that may be useful for anti-inflammatory...
studies in human cells. It is clear that upon intake, the polyphenols absorbed in the gut, are extensively modified.\(^8\)\(^8\) The bioavailability of polyphenols differ greatly among various compounds (reviewed in ref. 89). Several in vivo and clinical studies have demonstrated that plant polyphenols penetrate the blood-brain barrier in substantial amounts.\(^9\)\(^9\)\(^9\)

**Figure 4.** MTT assay indicates no significant cell death with LPS or OA\(^\beta\) treatment +/- plant extracts at a 1:100 dose. The MTT assay, specifically an assay of mitochondrial function, was performed on mixed glial cultures from APOE4-TR mice, the most vulnerable genotype, treated with LM-treated plant extracts (1:100, 150 μg/ml) and an inflammatory stimuli (LPS or OA\(^\beta\)), demonstrates no significant cell death. Data expressed as % control for LPS or OA\(^\beta\), n = 3 in duplicate for each experiment, expressed as means ± SEM.

**Figure 5.** Plant extracts dose-dependently attenuate APOE-modulated LPS-induced neuroinflammation (APOE-KO > APOE4 > APOE3 ≥ APOE2). LPS-induced TNF\(\alpha\) secretion from mixed glial cultures from APOE-TR or APOE-KO mice treated with dilutions (log scale: 1:100 to 1:5000) of plant extracts (xpf3, prn1, cop1, adt3 and wt). Plant mutant xpf3 dotted line (×), adt3 solid line (●), wt solid line (■), cop1 solid line (▲), and prn1 as solid line (♦). TNF\(\alpha\) secretion was measured as modulated by APOE (APOE-KO > APOE4 > APOE3 ≥ APOE2). LPS (100 ng/ml) was added 1 h after addition of plant extracts, and TNF\(\alpha\) levels in the media measured after 16 h by ELISA. Data are expressed as %LPS vehicle control (VC) for APOE3; values are an average of triplicates from a single experiment. Inset: enlargement of APOE2 and APOE3 cultures.
amounts following oral administration\(^90\). In the current *in vitro* study, plant extracts were treated with LM to obtain metabolites potentially active *in vivo*. LM contains enzymes like cytochrome P450s (CYP) that metabolize the plant extracts via hydroxylation and oxidative methylation\(^91–93\). Hence, the metabolic activation by LM mimic

Figure 6. *APOE-genotype* modulates LPS-induced neuroinflammation with *xpf3*-induced TNF\(\alpha\) reduction significant with *APOE4*. LPS (100 ng/ml)-induced TNF\(\alpha\) secretion from mixed glial cultures from *APOE2-, APOE3- or APOE4-TR* mice treated with wt or *xpf3* plant extracts at two dilutions (1:100 and 1:1000), or LPS from *Rhodobacter sphaeroides* (LPS-RS), a natural TLR4 antagonist used as a positive control. LPS was added 1 h after addition of plant extracts, and TNF\(\alpha\) levels in the media measured after 16 h by ELISA. Data expressed as TNF\(\alpha\) levels (pg/ml); n = 4 in triplicate for each experiment, expressed as means ± SEM. Entire genotype effect measured via one-way ANOVA with Tukey’s post-hoc test. Differences within treatment group or within genotype measured via two-way ANOVA with Bonferroni post-hoc test. \(p < 0.05\) denoted by: * vs. VC within genotype, § vs. *APOE4* and ¶ vs. wt (1:100).

Figure 7. Plant extracts dose-dependently attenuate *APOE*-modulated o\(\alpha\)\(\beta\)-induced neuroinflammation (*APOE-KO > APOE4 > APOE3 ≥ APOE2*). o\(\alpha\)\(\beta\) (10 \(\mu\)M)-induced TNF\(\alpha\) secretion from mixed glial cultures from *APOE-TR or APOE-KO* mice treated with dilutions (log scale: 1:100 to 1:5000) of plant extracts (*xpf3, prn1, adt3* and wt) demonstrate a dose dependent decrease in TNF\(\alpha\) secretion modulated by *APOE* (*APOE-KO > APOE4 > APOE3 > APOE2*). Plant mutant *xpf3* dotted line (×), *adt3* solid line (+), wt solid line (●), *cop1* solid line (▲), and *prn1* as solid line (♦). o\(\alpha\)\(\beta\) was added 1 h after addition of plant extracts, and TNF\(\alpha\) levels in the media measured after 16 h by ELISA. Data are expressed as % o\(\alpha\)\(\beta\) VC for *APOE3*; values are an average of triplicates from a single experiment. Inset: enlargement of *APOE2 and APOE3* cultures.
Aspects of physiological ingestion and metabolism, and produce metabolites that may survive in vivo. This is important as some compounds lose their activity post-liver processing and metabolism, while others may assume an active form.

These data provide evidence that enhancing the anti-inflammatory properties of A. thaliana via polyphenols and associated molecules have potential as dietary supplements as part of a therapeutic approach to neurodegenerative disorders. Furthermore, incorporating analogous mutations into consumable plants could benefit patients at a higher risk for AD. Acylation of several phenylpropanoids is typically seen in plant metabolism, dependent upon environmental cues, and produces many compounds of potential importance to medicine, which may have been promoted by our growth methods in young seedlings to promote a stress-resistant chemotype. Chemicals like 1,2 di-cine, which may have been promoted by our growth methods in young seedlings to promote a stress-resistant seed. HCAA have been implicated in oxidative stress defence in plants and utilized as a potential antioxidant, acid conjugated to a polyamine, referred to as hydroxycinnamic acid amide (HCAA), accumulates in the negative control with impaired polyphenol production. N,N'-di-sinapoyl spermidine is a hydroxycinnamic acid amide conjugated to polyamine, referred to as HCAA, accumulates in the negative control with impaired polyphenol production. N,N'-di-sinapoyl spermidine, while a minority chemical in an already potent flavonoid-based extract, was elevated post LM treatment in specifically seed97. HCAA have been implicated in oxidative stress defence in plants and utilized as a potential antioxidant.

Figure 8. APOE-genotype modulates α3-induced neuroinflammation with xpf3-induced TNFα reduction significant with APOE4. αβ (10 μM)-induced TNFα secretion from the mixed glial cultures from APOE2-, APOE3- or APOE4-TR with wt or xpf3 plant extracts at two dilutions (1:100 and 1:1000), or LPS-RS. αβ was added 1 h after addition of plant extracts, and TNFα levels in the media measured after 16 h by ELISA. Data expressed as TNFα levels (pg/ml); n = 4 in triplicate for each experiment, expressed as means ± SEM. Entire genotype effect measured via one-way ANOVA with Tukey’s post-hoc test. Differences within treatment group or within genotype measured via two-way ANOVA with Bonferroni post-hoc test. p < 0.05 denoted by: *vs. VC within genotype, §vs. APOE4 and ¶vs. wt (1:100).

An important and fundamental question regarding apoE is whether the presence of apoE4 causes a loss of positive function or a gain of toxic function. For development of future therapeutics, it is critical to understand whether apoE4 represents loss of positive function or gain of toxic function. If apoE4 is a gain of toxic function, then apoE4 will induce a detrimental response compared to a lack of apoE, experimentally defined as a functional improvement in APOE-KO mice compared to APOE4-TR mice. If apoE4 is a loss of positive function, the prediction is a neutral or improved response in APOE4-TR mice compared to APOE-KO mice. Thus, a comparison between apoE4 and apoE3 for a given function is not sufficient to determine if apoE4 is worse vs. not as effective compared to apoE3. The necessary three-way comparison is APOE-KO vs. APOE4 vs. APOE3. In this culture model, both LPS and αβ induce neuroinflammation that is APOE-modulated, with TNFα secretion. APOE-KO > APOE4 > APOE3 > APOE2, suggesting that APOE4 confers a loss of positive function. This is consistent with previous studies of both LPS and αβ-induced neuroinflammation in vivo102 and in vitro data49. The effect of the apoE isoforms on activation of glial cells can vary based on the cellular composition of the culture model. In vitro data from astrocytes demonstrate that LPS-induced secretion of proinflammatory cytokines including TNFα, IL1β and IL6 is APOE-modulated, with the levels of the cytokines higher in APOE2 compared to APOE3 and APOE4102. In contrast, levels of proinflammatory cytokines from LPS-induced microglial cultures are higher in APOE4 cultures, compared to APOE3 and APOE2103. Mixed glial cultures serve as a physiologically relevant model as it includes the individual activation of the microglia and astrocytes, as well as
the paracrine-like signaling between these two major immune cells in the brain. Indeed, studies in mixed glial cultures demonstrate that LPS-induced neuroinflammation is APOE-modulated (reviewed in ref. 56), with levels of proinflammatory markers (TNFα, IL1β, NO, IL6, etc.) higher in APOE4 than APOE397–98. In addition, mixed glial cultures from APOE-KO mice showed higher LPS-induced NO production compared to glial cells from APOE3-TR mice104. Importantly, in microglia/neuron co-cultures, LPS-induced loss of synaptic protein follows the order: APOE4 > APOE3 > APOE2, recapitulated in hippocampal slice cultures56.

One pathway through which APOE4 may modulate neuroinflammatory pathways is via TLR4-dependent signaling. Indeed, our published data in APOE/FAD-Tg mice demonstrate that microgliosis and astrogliosis are greater with APOE4 compared to APOE359. In addition, inflammatory mediators, as a result of TLR4 activation, are increased with APOE4 compared to APOE3. Complementing these in vivo findings, our in vitro data reveal that stimulation of TLR4 in mixed glial cultures with LPS or oAβ induces a similar neuroinflammatory response, as characterized by secretion of TNF-α, an effect more pronounced with APOE4 than APOE3. Importantly, both LPS- and oAβ-induced responses are inhibited by LPS-RS. Recent in vitro data also demonstrate potent TLR4 antagonism of oAβ-induced responses using the synthetic small molecule IAXO (Innaxon), developed at the University of Milano-Bicocca, and shown to inhibit innate or auto-inflammatory processes108–108.

These data can be used to further understand how to potentiate polyphenols in seedlings, and aid in the quest to develop plant-based materials suitable to prevent chronic inflammation. Furthermore, by studying the further actions of the phytochemicals, it may be possible to identify their targets and understand how they may exert their actions, as has been done for individual flavonoid chemicals in vitro109. Importantly, nutraceuticals are an inexpensive and effective option that could be potentially used to prevent neuroinflammation, hence delaying the onset of AD, specifically for APOE4-carriers, who are at a higher risk of both AD and non-AD APOE4-associated neuroinflammatory processes, including recovery from head injury, cerebral haemorrhage, stroke and cerebral artery amyloidosis.

Conclusions
We developed a protocol for growth and extraction of high-yielding polyphenols from A. thaliana mutant seedlings. An in vitro screen using a human apoE-relevant primary glial cell model demonstrated that mutant xpf3 (150 μg/ml) has the highest anti-inflammatory effect, as measured by TNFα levels in the media, compared to wt (150 μg/ml). Analysis of the ethanol extractable mutant-seedling chemicals indicated that xpf3 possessed the highest polyphenol content per tissue mass, and had a specific polyphenol profile. In addition, the findings demonstrate that APOE4 imparts a loss of positive function, compared to APOE-KO. Furthermore, oAβ induced an inflammatory response, similar to LPS, that was attenuated by LPS-RS, suggesting this response was via the TLR4 pathway. Together, this study demonstrated the use of extracts from mutant A. thaliana grown to maximize the polyphenol content as a model to identify plant targets that optimize the preventative strategy for AD patients, particularly for those at high risk for AD from the APOE4 allele.

Methods

Chemicals. All chemicals for plant material growth and extract preparation, unless otherwise noted, were obtained from Sigma (St. Louis, MO). A342 was purchased from California Peptide (now Echelon Biosciences, Salt lake city, UT; Cat. No. 641–15).

Plant accessions. Seeds of wt Columbia (Col) A. thaliana, and Col mutants carrying a T-DNA insertion within the coding region PRIN1 (PRN1; At3g59220, SALK_006963), EXCISION REPAIR ENDONUCLEASE HOMOLOGUE 3 (XPF3; At5g41150, SALK_096156), Constitutive Photomorphogenic1 (COP1; At2g32950, SALK_022133), AROGENATE DEHYDRATASE_3 (ADT3, AT2G27820; SALK_029949) were obtained from the Arabidopsis Biological Resource Center (Columbus, OH)110. The mutant lines are homozygous null for the reported insertions. Plants intended for seed stocks were grown as described11. For this study, three mutants of A. thaliana (xpf3, prn1 and cop1) were selected based on UV-C survival (indicating polyphenol production). adt3 was used as a negative control (produces low levels of phenylpropanoids12).

Plant growth conditions for experiments. Seeds of A. thaliana wt or T-DNA insertion mutants were prepared, sown, and grown (seedlings are 6–7 d) on 0.5 X Murashige and Skoog media 0.8% agarose phytatrays, then sealed in black Plexiglas boxes as described11. Cold-vernalized phytoatrays were then moved to continuous darkness (Dc) at 20 °C for 6 d. Dc seed sets were exposed to two sublethal UV treatments of 254 nm for 3 minutes (min), 315 nm (4 min) (radiation sources described in ref. 73), with 1 min between treatments, then phytoatrays were returned to darkness for 12 h. Sets of live seedlings were harvested immediately after irradiations for oxidative stress studies (see oxidative stress assays), or quick frozen in liquid nitrogen for extraction. For seedling survival assays (sublethal UV-B dose and lethal UV-C dose), seedlings were treated with 254 nm as described in11, and survival was determined by those seedlings standing and growing 24 h post-treatment, as described73. Those seedlings that “lodged” or fell over on the plate are characterized by major damage to the cells of the shoot and cotyledons and by 24 h and later, do not continue growing73.

Oxidative stress assays. 6-d-old seedlings received UV treatments (described above) or control (no UV treatment). Following the treatment, live seedlings were immediately harvested into a solution of the cell-permeable fluorescent probe CellRox Deep RedTM, with the ROS (Life Technologies) reagent dissolved as directed into anhydrous DMSO, for 30 min incubation under rotation (50 rpm/min rotation) in darkness. Plant material was washed with PBS for 3 min (four times) at room temperature then mounted without fixation for microscopy. The fluorescent signal from CellRox Deep Red was detected by CYS LED (see Microscopy), and both DAPI and FITC Ex/Em data were collected for phenylpropanoid florescence.
Microscopy. Seedlings were viewed on a Zeiss Observer.Z1 (ApoTome 2) deconvoluting microscope. Images were collected with Xcite 120 LED/Lumen Dynamics filter sets (CY5, DAPI, FITC) managed by Zen pro software (2012). Images were collected at the same exposure (control and experimental samples). At least 20 seedlings were viewed per experimental replicate. In post-processing software, fluorescence intensity was measured from at least 10 cells per cotyledon of a representative 5 imaged.

Plant materials extraction. Aerial portions ground in liquid nitrogen were weighed, and extracted with 95% aqueous ethanol by sonication (20 ml of solvent per gram of plant material, performed twice, 2 h each treatment). After filtration, the filtrate was evaporated to remove solvent using a rotavapor under reduced vacuum, then freeze-dried. The extract was then resuspended in sterile DMSO at a constant concentration (150 mg/ml) across the plant mutant samples.

Mixed glial cultures. Primary glial cultures (~95% astrocytes, 5% microglia) were prepared from the cerebral cortex of 1–2d old neonatal APOE-TR mice or APOE-KO mice as described. Briefly, cells from APOE2-TR, APOE3-TR, APOE4-TR or APOE-KO mice were harvested and on day in vitro (DIV) 10–12, when the cells were confluent, they were trypsinized and plated into two 175-cm² tissue culture flasks (passaged). On DIV 17–19, the secondary glial cells were plated into 24-well plates for treatment, as described below.

Liver microsomal treatment. The plant extracts from A. thaliana mutants were digested using mouse LM (Gibco, Life Technologies, Grand Island, New York) to mimic mouse ingestion, as described. Briefly, an NADPH generating system was established to provide the NADPH required by the P450 enzymes in the LM. The 200 μl reaction mixture (10 μl LM, 20 μl plant extract (1:1), 1.2 μl MgCl₂ (1 M), 6.6 μl NADP (10 mM), 6.4 μl glucose-6-phosphate (250 mM), 4.0 μl glucose-6-phosphate dehydrogenase enzyme (500 U/ml) in 152 μl HEPES buffer) was incubated at 37 °C for 30 min, followed by incubation on ice for 30 min to stop the reaction, and then centrifuged (2800 rpm, 10 min, 4 °C). The supernatant consisting of the plant extract metabolites (1:10, 15 mg/ml) potentially active in vivo were used for the in vitro treatments/screen. DMSO diluted in HEPES buffer was treated with LM, and the supernatant used as LM-vehicle control (LM-VC). The LM-VC was also used as the diluent for the plant extract dilutions.

HPLC and Mass Spectrometry (MS). Ten replicates of each mutant and wt were assessed by absorbance spectrum and by HPLC for prominent peaks (before LM treatment), to evaluate reproducibility of independently grown wt and mutant samples. Data were based on the HPLC chromatogram under UV at 320 nm. Average values from 10 batches of each plant genotype were reviewed to identify peaks. Further analysis was done by MS/MS, particularly for samples before and after LM treatment. The most effective mutant at higher dilution. From 10 batches of each plant genotype were reviewed to identify peaks. Further analysis was done by MS/MS, particularly for samples before and after LM treatment. The most effective mutant at higher dilution.

LPS and oAβ treatment. Secondary glial cells were seeded into 24-well tissue culture plates at a density of 1.44 × 10⁶ cells per plate. After 24 h, cells were washed with PBS to remove serum, and incubated in serum-free α-MEM media for an additional 24 h prior to treatment. The LM-digested plant extracts (xpf3, prn1, cop1, ad3 and wt) or LM-VC were added to the glial cultures at the indicated dilutions, ranging from 1:100 (150 μg/ml) to 1:5000 (3 μg/ml). After 1 h, LPS (100 ng/ml, Sigma Aldrich) or oligomeric preps of Aβ342 (10 μM), prepared as described, were added to the glial cultures to induce inflammation. LPS-RS (LPS from Rhodobacter sphaeroides, 10 μg/ml, Invivogen), a commercially available TLR4 antagonist, was used as a positive control. After 16 h of incubation, conditioned media from the 24-well plates was removed, flash frozen and stored at −80 °C. TNFα levels in the media were measured using a mouse TNFα ELISA (Invitrogen), following the manufacturer’s protocol. The TNFα levels are expressed as %LPS VC, or oAβ VC of each APOE genotype relative to the APOE3 VC.

MTT Assay. The MTT assay, a measure of cell viability that specifically measures mitochondrial function, was performed on mixed glial cultures isolated from APOE4-TR mice, the most vulnerable APOE genotype. The MTT assay was performed in duplicate, n = 3 independently replicated experiments. For Figs 5 and 7, the experiments were performed in duplicate to obtain dose curves for LPS- and oAβ-induced inflammation using the five plant extracts (xpf3, prn1, cop1, ad3 and wt), at five dilutions (log scale: 10−10 to 10−3).

Statistical analysis. Statistical significance was determined using GraphPad Prism version 5 for Macintosh. For microscopy data of Fig. 1, Graphpad was used for fluorescence measurement values, using two-tailed t-test with Welch’s correction. For Fig. 4, the MTT assay was performed in duplicate, n = 3 independently replicated experiments. For Figs 5 and 7, the experiments were performed in duplicate to obtain dose curves for LPS- and oAβ-induced inflammation using the five plant extracts (xpf3, prn1, cop1, ad3 and wt), at five dilutions (log scale: 10−10 to 10−3).
For Figs 6 and 8, data are presented as mean ± SEM; experiments were performed in triplicate, n = 3–4 independently replicated experiments (described in Fig. Legends). Entire genotype effect measured via one-way ANOVA with Tukey’s post-hoc test. Differences within treatment group or within genotype measured via two-way ANOVA with Bonferroni post-hoc test. p < 0.05 denoted by * vs. VC within genotype, † vs. APOE4 and ‡ vs. wt (1:100).

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Author Contributions
This study was a collaboration between three labs (PIs: M.J.L., K.M.W., C.-T.C.), M.J.L., L.T., K.M.W. and C.-T.C. planned the study and all the experiments. K.M.W. grew the plant seedlings and provided the raw plant extracts. The extracts were further processed and analyzed by M.Z. and C.-T.C., who performed chemical analysis on the extracts by HPLC, NMR and MS. M.J.L., L.T. and S.G. developed the protocol for liver microsome treatment, prepared the primary cells and performed the bioassays. M.J.L., L.T., S.G. and N.C. performed data analyses on the data from the bioassays. M.J.L., L.T., K.M.W. and S.G. were responsible for the manuscript preparation and preparation of the figures.

Additional Information
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