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ORIGINAL ARTICLE

Reducing chlorophyll levels in seed-filling stages results in higher seed nitrogen without impacting canopy carbon assimilation

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Abstract

Chlorophyll is the major light-absorbing pigment for plant photosynthesis. While evolution has been selected for high chlorophyll content in leaves, previous work suggests that domesticated crops grown in modern high-density agricultural environments overinvest in chlorophyll production, thereby lowering light use and nitrogen use efficiency. To investigate the potential benefits of reducing chlorophyll levels, we created ethanol-inducible RNAi tobacco mutants that suppress Mg-chelatase subunit I (*CHLI*) with small RNA within 3 h of induction and reduce chlorophyll within 5 days in field conditions. We initiated chlorophyll reduction later in plant development to avoid the highly sensitive seedling stage and to allow young plants to have full green leaves to maximise light interception before canopy formation. This study demonstrated that leaf chlorophyll reduction >60% during seed-filling stages increased tobacco seed nitrogen concentration by as much as 17% while canopy photosynthesis, biomass and seed yields were maintained. These results indicate that time-specific reduction of chlorophyll could be a novel strategy that decouples the inverse relationship between yield and seed nitrogen by utilising saved nitrogen from the reduction of chlorophyll while maintaining full carbon assimilation capacity.

KEYWORDS
canopy photosynthesis, gene regulation

1 | INTRODUCTION

Closing the gap between yield potential and realised current yield of crops while maintaining nutritional quality is necessary to meet future global food demand (Long & Ort, 2010; Ray et al., 2013; Zhu et al., 2010). A significant component of the crop yield gap is lower than the theoretical efficiency of the photosynthesis of crops in the field (Ainsworth & Long, 2021; Ort et al., 2015). The efficiency of photosynthesis decreases as the amount of absorbed photosynthetically active radiation increases (Sinclair & Muchow, 1999; Slattery & Ort, 2015; Slattery et al., 2013), suggesting that for agricultural purposes, crop plants invest too many resources in light capture while underinvesting in light

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utilisation. A major evolutionary benefit of overinvestment in light capture is that shading potential competitors confers a selective advantage (Zhang et al., 1999); even when photosynthesis is light-saturated and thus cannot utilise additional light, intercepting more light prevents a potential competitor from receiving and benefitting from the light. However, this investment strategy is suboptimal for an agricultural monoculture (Denison et al., 2003; Loomis, 1993), where the goal is to maximise net primary productivity for the field. In agricultural canopies, radiation penetration, and therefore radiation use efficiency, is decreased by dense foliage at the top of the canopy, which absorbs most of the incident photosynthetically active radiation (Long, 1993; Long et al., 2006; Slattery & Ort, 2015). The rate of photosynthesis reaches saturation (A_{sat}) at a light intensity well below that of full sunlight. At moderate to high light intensities, the rate at which sunlight is absorbed by sun-exposed leaves vastly exceeds the amount needed to reach A_{sat}, and the excess absorbed photons are dissipated by photoprotective mechanisms and thereby wasted (Ort, 2001; Takahashi & Badger, 2011).

Modelling studies have proposed that plants produce excess chlorophyll, and thus, reducing chlorophyll production could benefit nitrogen use efficiency without compromising total canopy carbon gain. Using the sunlit-shaded model, Ort et al. (2011) proposed that a 50% reduction in chlorophyll would improve light distribution and increase canopy photosynthesis; however, further decreases in chlorophyll would be disadvantageous. Studies using advanced multi-layer canopy models have predicted that leaf chlorophyll could be decreased by 50% without penalty to canopy photosynthesis while additionally bringing about a potential 9% savings of leaf nitrogen (Walker et al., 2018). A separate modelling study concluded that a 60% reduction in chlorophyll could increase nitrogen use efficiency and increase canopy photosynthesis if the saved nitrogen were reinvested to increase photosynthetic capacity in areas of the canopy where light intensity was increased (Song et al., 2017). In addition, experimental evidence supports the premise that lowering leaf chlorophyll could be beneficial. Field-grown low chlorophyll rice and soybean show similar or greater photosystem II efficiency, Rubisco carboxylation rates, and nitrogen-use efficiency when compared to dark green wild-types (WTs) (Gu et al., 2017; Li et al., 2013; Sakowska et al., 2018). Low chlorophyll rice and tobacco mutants were reported to have higher yields than dark green WTs at higher planting densities (Gu et al., 2017; Kirst et al., 2017).

However, one predicted disadvantage of reduced leaf chlorophyll is that low chlorophyll content early in the season before canopy closure will result in reduced light interception efficiency because less light is absorbed by young leaves while more light is transmitted to the soil. Canopy modelling predicts that lowering chlorophyll will increase canopy photosynthesis after canopy closure and decrease photosynthesis before the closure (Long et al., 2006; Song et al., 2017). The benefits of lowering chlorophyll are expected to depend on both the timing and extent of chlorophyll reduction, which can be realised by time-specific gene regulation through inducible promoters. The ethanol-inducible gene expression system (Felenbok et al., 1988) found in fungi has been adopted in plant science (Caddick et al., 1998; Salter et al., 1998) and has previously been used to reduce chlorophyll synthesis in tobacco (Chen et al., 2003); thus it is a practical tool for realising time-specific chlorophyll regulation.

Reducing chlorophyll production could free nitrogen resources for other uses by the plant, possibly increasing seed nitrogen without compromising carbon assimilation. In cereals, the nitrogen content in seeds reflects the seed protein content and, thus the seed's nutritional quality (Good et al., 2004). Studies about the genetic basis for seed composition have revealed that most crops (Simmonds, 1995), including maize (Feil et al., 1990) and wheat (Canevara et al., 1994), as well as the rape seed (Brennan et al., 2000), have an inverse relationship between seed yield and protein concentration. This is proposed to occur because of the dilution of proteins by carbohydrates (Acreche & Slafer, 2009) and the competition between carbon and nitrogen metabolisms for energy (Munier-Jolain & Salon, 2005). Plants can use leaves for either carbon assimilation or nitrogen remobilisation, which are mutually exclusive processes (Havé et al., 2017).

Leaves capture solar energy for photosynthetic carbon assimilation and export carbon in the form of sugars to the seeds. Reduced photosynthetic activity during senescence decreases carbon assimilation and sugar export to seeds and other sinks. On the other hand, nitrogen is transferred from leaves to seeds primarily during senescence, as protein is broken down and the nitrogen mobilised (Masclaux-Daubresse et al., 2008). Delaying leaf senescence increases seed yield due to the maintenance of carbon assimilation, while the maintenance of the photosynthetic apparatus delays nitrogen remobilisation and decreases seed nitrogen content. For cereals and crops that are cultivated for their seed protein content, this constitutes a dilemma that opposes yield performance against seed nitrogen (Distelfeld et al., 2014; Good et al., 2004; Gregersen et al., 2013; Oury & Godin, 2007; Uauy et al., 2006). Perhaps saved nitrogen from decreased chlorophyll production could increase seed nitrogen without penalty on yield because more free nitrogen is available in the seed-filling stages without compromising canopy photosynthesis, as models have predicted (Ort et al., 2011; Song et al., 2017; Walker et al., 2018).

The main objective of this study was to investigate the impact of reducing chlorophyll in a time-specific manner, particularly in later plant developmental stages at the start of seed filling, using a combination of field and greenhouse work. We hypothesised that the reduction of leaf nitrogen sequestration in chlorophyll during seed fill would increase seed nitrogen without a reduction in net carbon assimilation. We predicted that lowering chlorophyll at a later developmental stage would not decrease and possibly would increase canopy photosynthesis, biomass and seed yield. To test these hypotheses, we used transformed tobacco (*Nicotiana tabacum* cv. Petite Havana) to generate small RNAs (sRNAs) to downregulate Mg-chelatase subunit I (*CHLI*) expression via an ethanol inducible promoter, leading to the reduction of chlorophyll only when ethanol was applied. Mg-chelatase, consisting of three subunits (I, D, H), catalyzes the insertion of Mg^{2+} into protoporphyrin IX during chlorophyll synthesis (Figure 1a). When both Mg^{2+} and ATP are present, the *CHLI* subunit forms a protein complex with the *CHLD* subunit and stabilises an active Mg-chelatase (Farmer et al., 2019; Lundqvist et al., 2010). *CHLH* subunit is a porphyrin-binding subunit and catalyzes magnesium insertion via glutamate residue (Adams et al., 2020;

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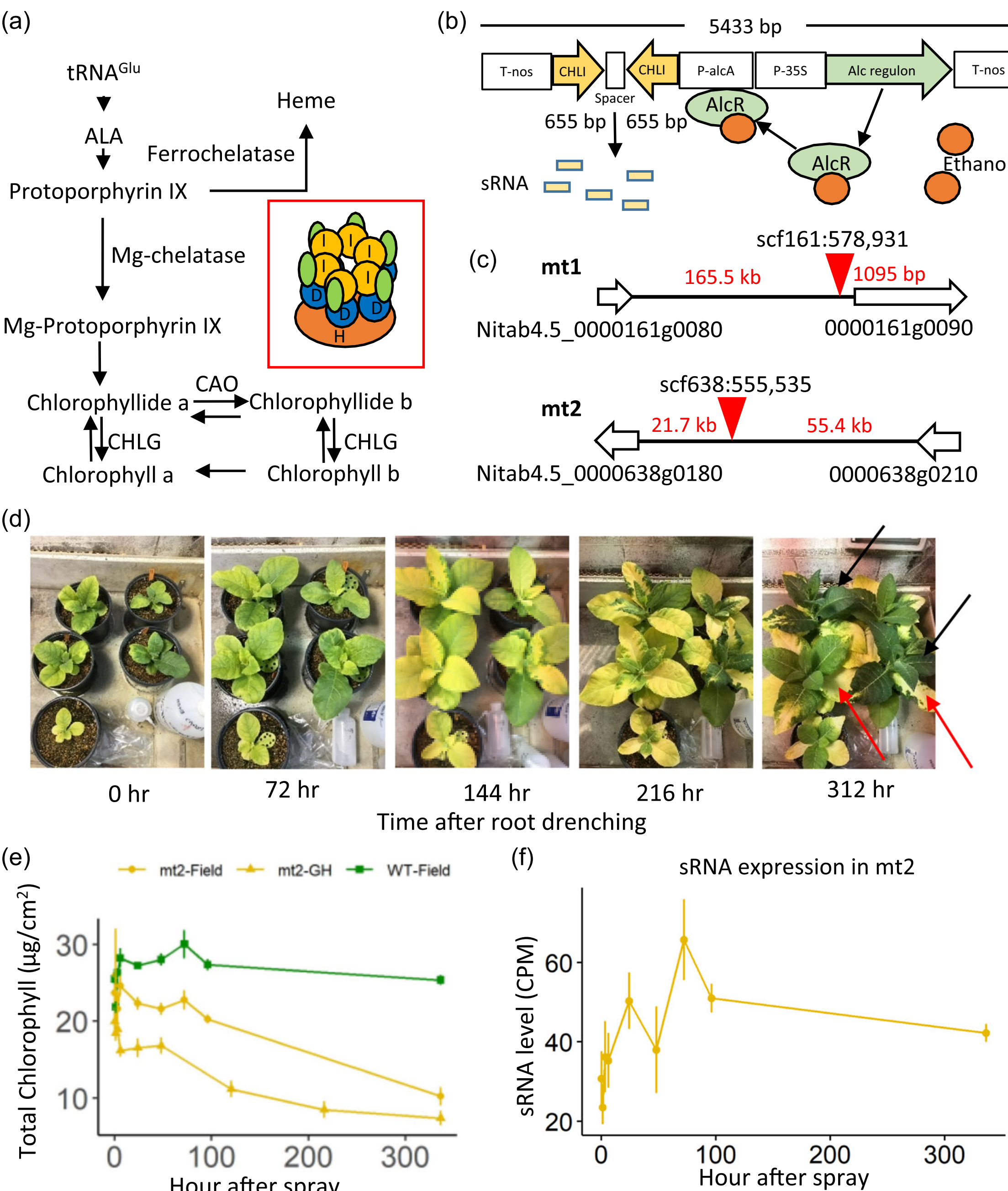

FIGURE 1 Developmental stage-specific downregulation of chlorophyll using ethanol inducible promoter. (a) Biosynthesis pathway of chlorophyll and the model structure of Mg-chelatase with three subunits (red rectangle). (b) Design of ethanol inducible CHLI sRNA construct. (c) Location of T-DNA (red triangle) in two tobacco mutants (mt1 and mt2). (d) Changes in leaf colour phenotype after 100 mL 1% ethanol applied to roots in the greenhouse (mt1). Newly developed leaves are fully dark green (black arrow), while affected leaves and partial leaf area (red arrow) did not recover from the low chlorophyll phenotype. (e) Levels of total chlorophyll after 2% ethanol spray to leaves of wild-type and mt2 in the greenhouse and 2019 Illinois field. (f) The level of CHLI sRNA expression (CPM, counts per million). Error bars represent standard error ($n = 4$).

Willows et al., 1996). We initiated chlorophyll reduction later in the plant's growth stage at the start of seed filling, which allowed young plants to have full green leaves to maximise light interception before the canopy was formed when losses due to light inhomogeneity were small. This study demonstrated that a reduction of leaf chlorophyll by more than 60% after canopy closure at the start of seed filling resulted in as much as a 17% increase in seed nitrogen concentration with no change (i.e., reduction or increase) in biomass or seed yield.

2 | MATERIALS AND METHODS

2.1 | Cloning and transformation

The constructs were generated using Golden Gate Cloning (Engler et al., 2008, 2009). Level 0 constructs, including *alcR* regulon (EC27885), *alcA* promoter (EC27886), *GA20* intron (EC27888) and *CHLI* RNAi (EC27891) were newly synthesised based on the sequence

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used in Chen et al. (2003). The assembled construct is described in the Results section (Figure 1b). *N. tabacum* cv. Petit Havana was genetically transformed using *Agrobacterium tumefaciens*-mediated transformation (Gallois & Marinho, 1995). Twenty-two independent T0 transformations were generated to produce T1 progeny. T-DNA copy number was determined on T1 plants through copy number quantitative polymerase chain reaction (qRT-PCR) analysis (iDNA Genetics) (Table S1). From these results, two events were selected based on the leaf colour after ethanol treatment in the greenhouse. Then, the two selected lines were selfed and produced T2 progeny with copy numbers confirmed for the homozygous single insertion (Table S1). T3 progeny carrying a homozygous single insertion were used in the experiments.

2.2 | T-DNA location

Genomic DNA was extracted from young leaves, as described in Cho et al. (2019). The locations of T-DNA in two mutants were revealed by targeted locus amplification as performed by Cergentis B.V. (de Vree et al., 2014). The location of T-DNA in mt1 was confirmed by PCR using a primer set; the forward primer resides on the native tobacco genome near the breakpoint, and the reverse primer resides on the T-DNA. The location of T-DNA in mt2 was confirmed by using the GenomeWalker kit (Clontech Laboratories) following the manufacturer's recommended protocols. Both locations were confirmed by PCR using unique primer sets. All primer sequences are described in Table S4.

2.3 | Greenhouse and field experiments

Plants were grown as described by South et al. (2019) for greenhouse experiments and Kromdijk et al. (2016) for field experiments. Details about greenhouse and field experiments were described in the Results section, Figures S3–S7, S11–S12 and S14 and Supporting Infomation Materials and Methods.

2.4 | Ethanol treatment

For the root drenching, 100 mL of 1% (vol/vol) ethanol was applied to the roots (Figure 1d). For all leaf spraying, fresh 2% (vol/vol) ethanol was prepared every morning in both greenhouse and field experiments. A hand-held sprayer (Chapin 26021XP 2-Gallon ProSeries Poly Sprayer) was used until the sun leaves (four-fifths leaf from the top, exposed to full sunlight) were fully soaked with the solution. Spray time was between 7 and 9 AM, or after 5 PM to avoid full sunlight.

2.5 | Chlorophyll measurement

Soil plant analysis development (SPAD) measurements for chlorophyll content were taken at the same spot on the leaf as the gas exchange measurements for the photosynthetic response curves. To convert SPAD readings to chlorophyll content using a linear function (Figure S15), we collected leaf disks 1 cm in diameter (the same area measured by SPAD) in one field (Illinois in 2019 [IL2019]) and greenhouse experiment, which were frozen in liquid nitrogen for at least 10 min and stored in the freezer (−80°C) until they were lyophilised (Benchtop Freeze Dryers; Labconco Co.). Chlorophyll content was then determined using 100% ethanol extraction (Ritchie, 2006) and analysed with a spectrophotometer (BioTek PowerWave Microplate Reader; Agilent). For other experiments, chlorophyll content was measured by SPAD (Chlorophyll Meter SPAD-502 Plus; Konica Minolta) on a sun leaf. Three (before ethanol treatments) to nine (after ethanol treatments) plants per plot were measured in all field experiments, and every plant was measured in the greenhouse.

2.6 | sRNA extraction and next-generation sequencing analysis

Leaf disks 1 cm in diameter were collected from the same leaf at multiple time points: 0, 1, 3, 6, 24, 48, 72, 96, and 336 h after the first ethanol spray. Leaf discs were immediately frozen in liquid nitrogen for at least 10 min and stored in the freezer (−80°C) until they were lyophilised. Total RNA was isolated from freeze-dried leaf disks using phenol–chloroform extractions (Wang & Vodkin, 1994) and precipitated with ethanol but without lithium chloride to preserve sRNAs (Cho et al., 2013, 2017). The sRNA libraries were prepared using the NEBNext Small RNA Sample Prep kit (New England Biolabs). High-throughput sequencing was performed with NovaSeq-6000 (Illumina) by the Keck Center (University of Illinois) using Illumina protocols. Generally, a total of 8–10 million reads were obtained from these deep-sequencing libraries. Adapter-trimmed sequences were aligned to the tobacco genome (Edwards et al., 2017) and quantified by using the programme Salmon (Patro et al., 2017).

2.7 | A/C_i response curves

The response of A to C_i in randomly chosen sun and shade leaves was measured at a saturating light intensity of 2000 µmol quanta mol^{-2} s^{-1} by using a portable infrared gas analyser (LI-COR 6800; LI-COR). Illumination was provided by a red–blue fluorometer light source attached to the leaf cuvette. Measurements of A were started at the ambient CO_2 concentration (C_a) of 400 µmol/mol before C_a was decreased stepwise to a lowest concentration of 0 µmol/mol and then increased stepwise to an upper concentration of 1300 µmol/mol (400, 300, 200, 100, 50, 0, 400, 400, 600, 800, 1000 and 1300 µmol/mol). All parameters were calculated from A versus C_i by fitting the data to a non-rectangular hyperbola. The maximum carboxylation efficiency (CE), the CO_2 compensation point (Γ), the CO_2 saturated rate of A_{net} (A_{sat}), and the empirical curvature factor for the A/C_i curves (ω) were calculated from A/C_i measurements. The maximum

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rate of carboxylation (V_{cmax}) and maximum electron transport rate (J_{max}) were determined from these A/C_i curves using a curve fitting utility (Sharkey, 2016; Sharkey et al., 2007) with mesophyll conductance (g_m) of 0.57 mol m^{-2} s^{-1} bar^{-1} (von Caemmerer & Evans, 2015).

2.8 | A/Q_{abs} response curves

Photosynthesis as a function of light (A/Q_{abs} response curves) in randomly chosen sun and shade leaves was measured under the same cuvette conditions as the A/C_i curves mentioned above at an ambient CO_2 concentration (C_a) of 400 µmol/mol by using a portable infrared gas analyser (LI-COR 6800; LI-COR). The leaves were initially stabilised at a saturating irradiance of 2000 µmol quanta m^{-2} s^{-1}, after which A and g_s were measured at the following light levels: 2000, 1500, 1000, 750, 500, 200, 180, 160, 140, 120, 100, 80, 60, 40, 20 and 0 µmol m^{-2} s^{-1}. The measurements were recorded after A reached a new steady state (1–2 min) and before g_s stabilised to the new light levels. All parameters were calculated from A versus absorbed photosynthetic photon flux density (PPFD) by fitting the data to a non-rectangular hyperbola. A quadratic relationship between leaf light absorptance and chlorophyll level (SPAD) was determined using an integrating sphere (Jaz Spectroclip; Ocean Optics) as described in Walker et al. (2018) (Figure S13D). The maximum quantum yield of CO_2 assimilation (Φ_{CO2}), respiration rate (R), the light-saturated rate of net CO_2 assimilation (A_{sat}), the empirical curvature factor (θ), and the maximal photochemical yield of photosystem II obtained at low light ($\Phi_{PSII(LL)}$) were calculated from A/Q_{abs} curves.

2.9 | A' calculations

A simple three-leaf-layer concept was used to model canopy photosynthetic rates (Figure S13A), simulating a leaf area index (LAI) of three similar measurements from two field trials (Figure S8A). Modelled parameters included diurnal PPFD incident on the uppermost leaf layer (Figure S13B), linear relationships between transmittance and reflectance (Figure S13C), a quadratic relationship between leaf light absorptance, chlorophyll level (SPAD) (Figure S13D) and the photosynthesis parameters from the light response curves (A/Q_{abs} curves; Table 1). These parameters were used to calculate light absorbed and carbon assimilated throughout a 24-h period; the carbon assimilated in each leaf layer was summed to calculate the total net carbon assimilated by the canopy (A').

2.10 | Leaf composition analysis

Dried leaves from the harvested plants in the IL2019 field trial were collected and analysed to investigate the form of nitrogen they contained. Tissue samples were sent to a commercial lab (Midwest Laboratories) where the samples were ground, homogenised, and analysed for total nitrogen (Dumas method with a KECI FP428 nitrogen analyser, AOAC method 968.06), nitrate (NO_3) nitrogen (cadmium reduction automated FIA determination, AOAC 968.07), ammonia (NH_4) nitrogen (distillation method for the determination of ammonia nitrogen, AOAC 920.03) and organic nitrogen (Kjeldahl method, AOAC 2001.11). The same ground samples were independently analysed by an elemental analyser (see above for method) to calculate the C/N ratio.

2.11 | LAI and specific leaf area measurements

LAI was determined for four (IL2019) plants in each plot using a leaf area meter (LI-3100; LI-COR). With the measurement of leaf area, the dry biomass of the leaves was measured to calculate specific leaf area (SLA) = leaf area (m^2)/leaf dry mass (kg).

2.12 | Seed composition analysis

Dried seeds were homogenised and ground into a powder with a ball mill (Geno Grinder 2010; BT&C). Ground material was weighed into tin capsules for C and N analysis and combusted with an elemental analyzer (Costech 4010CHNS Analyzer; Costech Analytical Technologies, Inc.). Acetanalide and apple leaves (National Institute of Science and Technology) were used as standards as in Masters et al. (2016).

2.13 | Statistical analysis

For all experiments, with one exception, data were analysed as randomised complete-block designs with all factors treated as fixed effects, while blocks were considered as random effects (α = 0.05). For the SoyFACE experiment, data were analysed as a split-plot design. α was predetermined as 0.1 to minimise the potential for Type II errors, as per the standard practice for this specific field site (http://www.soyface.uiuc.edu). Models were analysed using mixed effects models using ANOVA with restricted maximum likelihood and Satterthwaite's estimate for degrees of freedom (R statistical software, version 4.0.5; lmer function from lme4 package, version 1.1-27.1; least square means differences using the difflsmeans function from lmerTest package, version 3.1-3). If the block effect was found to be nonsignificant, it was removed. In addition, if interactions were nonsignificant, they were also removed. When errors were not nonhomogeneous, White's adjustment was used. When errors were nonhomogeneous across factors, Wilcoxon's non-parametric rank test was used. Adjusted p values were used for multiple comparisons with Tukey's adjustment or, when appropriate, with Dunnett's comparison to the control (WT).

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3 | RESULTS

3.1 | Ethanol inducible RNAi mutant can suppress chlorophyll levels by downregulating *CHLI* in a developmental stage-specific manner

To decrease leaf chlorophyll, we designed an ethanol-inducible RNAi construct that targets *CHLI* (Nitab4.5_0006237g0030). *CHLI* is a known target enabling reduction of chlorophyll with minimal pleiotropic effect, as naturally occurring as well as mutagenized low chlorophyll soybean mutants with genetic disruption in *CHLI* grow as strongly as the isogenic WT parent (Campbell et al., 2015; Sakowska et al., 2018; Slattery et al., 2017). The 35S promoter constitutively expresses the *Alc* (alcohol dehydrogenase I) regulon (Sealy-Lewis & Lockington, 1984), which binds to the *alcA* promoter in the presence of ethanol (Figure 1b). We inserted the ethanol-inducible *CHLI* RNAi construct into tobacco and selected mutant 1 (mt1) and mutant 2 (mt2), which responded to ethanol treatment among 22 transformation events under greenhouse conditions. To assess the positional effect of T-DNA insertion in two mutant lines, we investigated the T-DNA copy number and its genomic location. Our selection process ensured that the mutant lines chosen were single-insert homozygous for the T-DNA locus (Table S1). The T-DNA integration site is located on scaffold 161:578,931 in mt1, while the T-DNA is located on scaffold 638:555,535 in mt2 (Figures 1c, S1, and S2). The nearest gene to the T-DNA insertion site is located 1095 bp away in mt1 and 21.7 kb away in mt2 (Figure 1c).

We evaluated various ethanol application methods suitable for field experiments. Initially, we applied 100 mL of 1% (vol/vol) ethanol to the roots of mt1 in the greenhouse. Leaves began to exhibit a low chlorophyll phenotype 72 h (3 days) after ethanol application (Figure 1d). Leaves that developed 216 h (9 days) after the ethanol application were completely dark green (Figure 1d, black arrows), indicating that the plants reinitiated chlorophyll synthesis once the effects of the RNA interference abated. In some cases, the whole leaf or partial area of a leaf (Figure 1d, red arrows) did not recover from the low chlorophyll phenotype even though the plant reinitiated chlorophyll synthesis in new leaves (Figure 1d). The root-drenching method had two major issues that made it impractical for field trials. First, chlorophyll in existing leaves was so greatly reduced that the leaves turned completely white; canopy modelling suggested that greater than a 70% reduction in chlorophyll would not be beneficial for canopy photosynthesis (Ort et al., 2011). Second, root drenching would not be practical for large-scale field trials. To overcome these limitations, we applied 2% ethanol spray to the leaves of WT, mt1 and mt2 plants in both greenhouse and field settings. In the greenhouse, this spray method showed noticeable responses in both mt1 and mt2 (Figures S3 and S4), albeit with slight phenotypic differences between the two mutant lines (Figure S5). However, under field conditions, mt1 did not respond to the ethanol spray, while mt2 responded consistently under both greenhouse and field conditions (Figure S6). Notably, the chlorophyll levels in mt2 began to decrease 120 h (5 days) after the initial ethanol spray, while WT chlorophyll levels remained unaffected (Figure 1e). The expression of *CHLI* sRNA increased 3 h after the first ethanol spray (Figure 1f), peaked at 72 h (3 days) and remained at a high level throughout the daily treatment regimen.

In summary, the spray method effectively reduced chlorophyll to a desirable level (50%–70% reduction compared to the WT) in the mt2 genotype under field conditions during the desired development stages.

3.2 | Reducing the chlorophyll level of leaves in the seed-filling stages resulted in higher nitrogen concentration in the seed without penalty to biomass or seed yield

In the greenhouse, we tested the hypothesis that saved nitrogen from chlorophyll production could be used for seed protein by subjecting the inducible RNAi tobacco mutant (mt2) to variable soil nitrogen: we chose four levels of insufficient nitrogen and one with an adequate supply. The 2% (vol/vol) ethanol spray started 60 days after planting (DAP) when flowering began (followed shortly by seed filling). The levels of chlorophyll at 84 DAP were significantly lower in the ethanol-sprayed mutants than the WT in all treatments (Figure 2a). Chlorophyll reduction in mutants was between 60% and 68% less in the nitrogen-deficient conditions and 54% less in the adequate nitrogen condition. Plants were harvested at 101 DAP; plant material was collected to measure dry biomass, and seeds were collected for weight and tissue composition. There was no difference in above-ground biomass or seed weight between WT and mutants for all treatments (Figure 2b,c). However, the seed nitrogen concentration was higher in low chlorophyll mutants under the 0.8 g urea (deficient) by 17% (Figure 2d). Seed carbon concentration remained the same in all treatments (Figure 2e). However, due to the increase in nitrogen, the carbon-to-nitrogen ratio (C/N) of the seeds was reduced by 8% in the nitrogen-deficient condition (Figure 2f).

Under field conditions using conventional fertilisation practices, we evaluated the impact of reducing the chlorophyll level of the leaves on seed nitrogen concentration. In 2021, at the SoyFACE experimental site, we compared the above-ground biomass, seed weight from the first flowering branch, as well as nitrogen and carbon concentration of seeds from mt2 sprayed twice daily and mt2 without an ethanol treatment. We observed a 35% decrease in chlorophyll concentration at 77 DAP in the ethanol-sprayed mutants relative to non-sprayed mutants (Figure 3a). Above-ground biomass and seed weights were not different between the two mutant treatments (Figure 3b,c). However, the nitrogen concentration of the seeds increased by 7% in the sprayed low chlorophyll mutants (Figure 3d). We observed a C/N ratio decreased by 7% (Figure 3f) and

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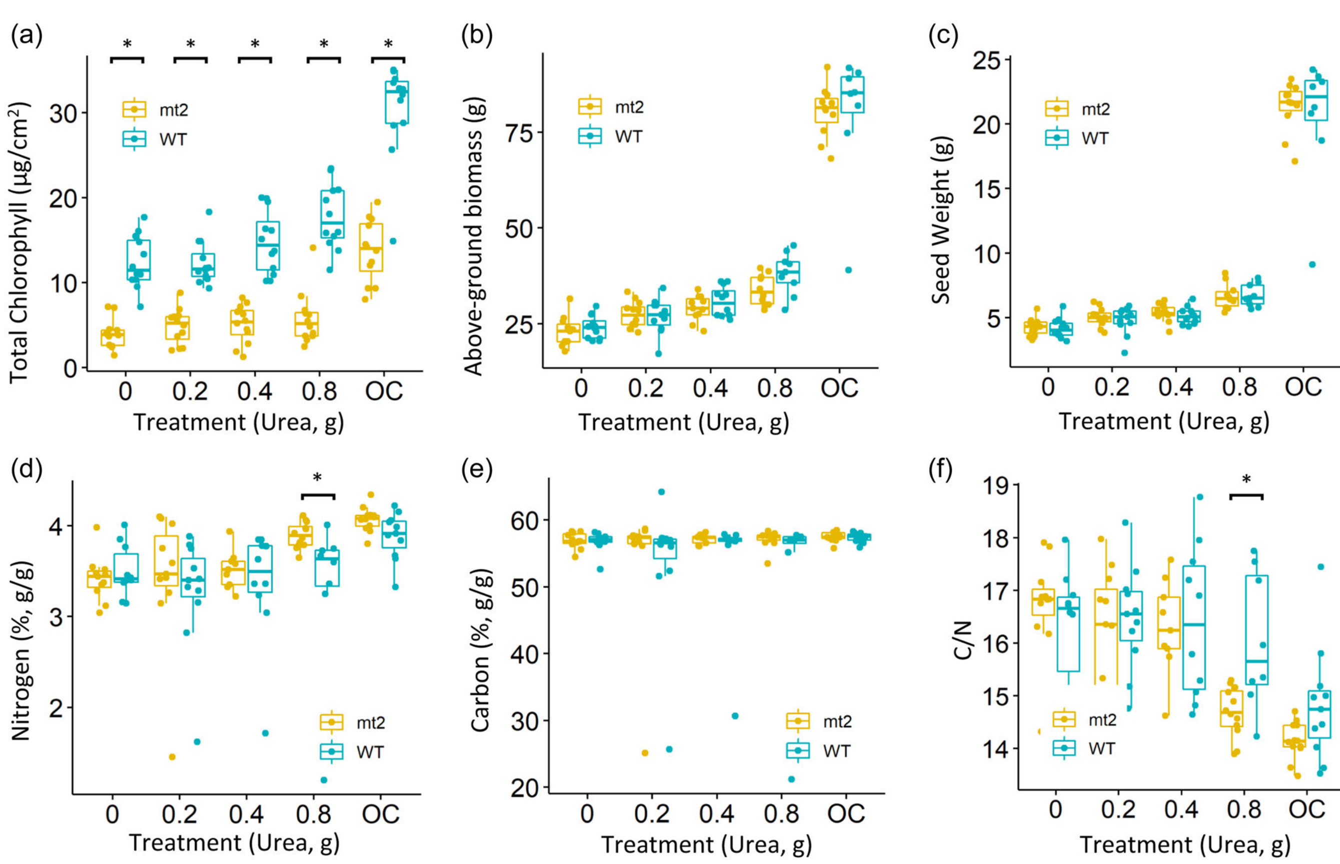

FIGURE 2 Increased nitrogen content and low C/N in seeds of low chlorophyll mutants in the greenhouse. Wild-type (WT) and mutant 2 (mt2) tobacco were treated with different amounts of fertiliser in the greenhouse. The *X*-axis shows the amount of urea added in a pot. OC stands for one teaspoon of Osmocote. (a) Total chlorophyll level 84 days after planting (DAP) and 24 days after treatment (DAT) ($n = 12$). (b) Above-ground biomass ($n = 12$). (c) Seed weight ($n = 12$). (d) Nitrogen concentration of seed ($n = 8–12$). (e) Carbon concentration of seed ($n = 8–12$). (f) C/N ratio of seed ($n = 8–12$). The box plots show the median (central line), the lower and upper quartiles (box) and the minimum and maximum values (whiskers). The statistical analysis was done using the Wilcoxon rank test ($\alpha = 0.05$). *Significant difference within treatment when present. [Color figure can be viewed at wileyonlinelibrary.com]

no change in the carbon concentration of the seeds (Figure 3e) between these treatments as well.

These results indicate that reducing the chlorophyll concentration of tobacco leaves in the seed-filling stages can increase the nitrogen concentration of seeds without decreasing biomass and seed production under greenhouse (mt2-2 vs. WT) and field (mt2-2 vs. mt2-0) conditions.

3.3 | Up to 62% chlorophyll reduction in later development stages resulted in no change of above-ground biomass in the field tests

To investigate the impact of reduced chlorophyll on canopy photosynthesis and biomass, we tested the ethanol-inducible RNAi mutants in three tobacco field trials: IL2019, Puerto Rico in 2019 (PR2019), and Illinois in 2020 (IL2020). In the Illinois 2019 field experiment (IL2019), we tested three genotypes: WT, mutant 1 (mt1), and mutant 2 (mt2). We had three treatments per genotype—spray once a day (−1), spray twice a day (−2), or no treatment (−0)—to evaluate the effect of the ethanol spray on the chlorophyll level of the canopy and on above-ground biomass. The plants were sprayed with 2% (vol/vol) ethanol from canopy closure (48 DAP) until 1 week before harvest (74 DAP). The low chlorophyll phenotype was easily discernible with the naked eye (Figures 4a and S7). We measured the level of chlorophyll in the canopy during the entire field season. The chlorophyll level in the mt2-2 (sprayed twice a day) plot began to decrease after 5 days of ethanol spraying and remained 30%–50% lower than the WT plot during the remainder of the season (Figure 4c; Table S2). Despite the reduction in chlorophyll, we observed no change in above-ground biomass between mt2 treatments (Figure 4b). Unlike the response that we observed in the greenhouse, mt1 did not respond to ethanol induction in the field, such that mt1-0, mt1-1, and mt1-2 showed no significant difference in chlorophyll level (Figures 4c, S6 and S7).

We investigated the nitrogen composition of leaves that we harvested at 81 DAP. Notably, the concentration of total nitrogen increased in the leaves of the mt2 genotype with the ethanol treatment, while the WT and mt1 showed no response (Figure 4d). Furthermore, the concentration of organic nitrogen showed a similar

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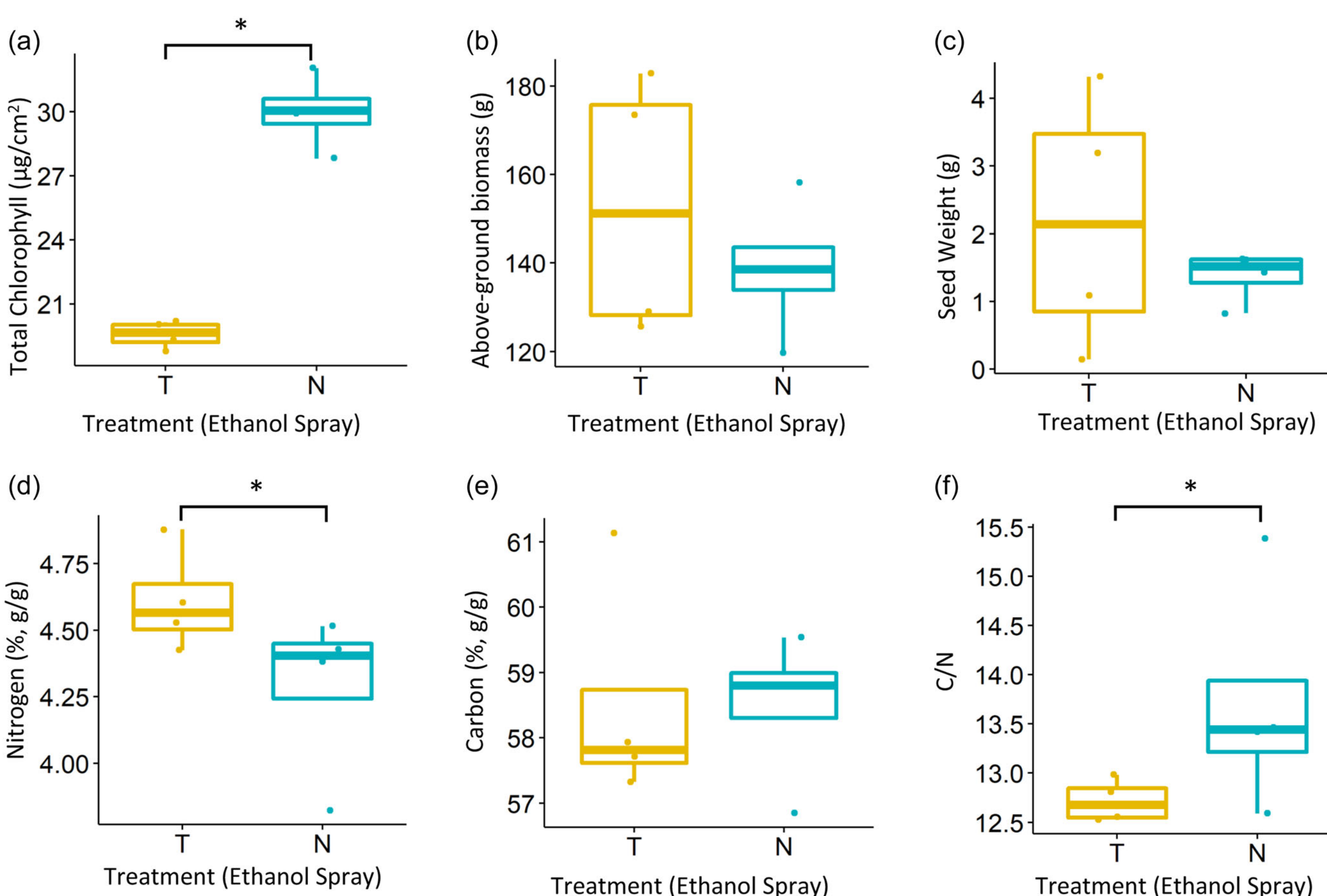

FIGURE 3 Increased nitrogen concentration and low C/N in seeds of low chlorophyll mutants under field conditions. Mutant 2 (mt2) tobacco was grown with or without ethanol treatment under field conditions. The *X*-axis shows the ethanol treatment (N, nontreatment; T, treatment). (a) Total chlorophyll level 77 DAP and 17 DAT. (b) Above-ground biomass. (c) Seed weight. (d) Nitrogen concentration of seed. (e) Carbon concentration of seed. (f) C/N ratio of seed. The box plots show the median (central line), the lower and upper quartiles (box) and the minimum and maximum values (whiskers). The statistical analysis was done using a linear mixed model ANOVA (n = 4 blocks, α = 0.1). *Significant difference within treatment when present. [Color figure can be viewed at wileyonlinelibrary.com]

pattern, an increase in the leaves of the mt2 genotype with the ethanol treatment, whereas the other two genotypes did not respond to the treatment (Figure 4e). LAI, SLA, as well as the concentrations of nitrate (NO_3) nitrogen and ammonia (NH_4) nitrogen, did not respond to the treatment in any genotype (Figures S8 and S9). Utilising an elemental analyzer with the same sample set, we observed a similar increase in nitrogen concentration (Figure S10), reaffirming the results (Figure 4d). We concluded that the ethanol spray had no impact on the above-ground biomass or chlorophyll levels of mt1 and WT genotypes. However, the ethanol spray led to a reduction in chlorophyll levels by up to 50% in mt2 and an increase in organic nitrogen concentration, with no observed changes in above-ground biomass.

The IL2019 trial revealed no effect of sprayed ethanol on the above-ground biomass of control full green genotypes, so we focused on the comparison of chlorophyll reduction and above-ground biomass in successive field trials. To assess this effect, three genotypes (WT, mt1 and mt2) were tested with one spray treatment twice daily with 2% (vol/vol) ethanol in PR2019. A 58% decrease in canopy chlorophyll level was observed in the mt2 genotype after treatment (Figure S11A–C; Table S2), while the above-ground biomass was not significantly different (Figure S11D). In the IL2020 field trial, only WT and mt2 genotypes were used as mt1 plants did not respond to the sprayed ethanol treatment and had similar chlorophyll content as WT plants in previous field trials (Figures 4c, S6, S7 and S11; Table S2). There was no difference in above-ground biomass between any treatment (WT-2, mt2-2, mt2-0 in Figure S12C), while up to a 62% chlorophyll reduction was observed in mt2-2 (Figure S12A,B,D–F,G; Table S1).

Cumulatively, the evidence from these three-field trials demonstrated that a twice-daily canopy spraying of ethanol effectively reduced chlorophyll content in mt2 tobacco by up to 62%, with the magnitude of the response varying between year and location. Perhaps more importantly, these trials demonstrated with repeatability that large reductions in chlorophyll after canopy closure had no effect on the above-ground biomass of tobacco. [We did not collect seeds from any of these field trials due to Animal and Plant Health Inspection Service regulations regarding seed dispersal.]

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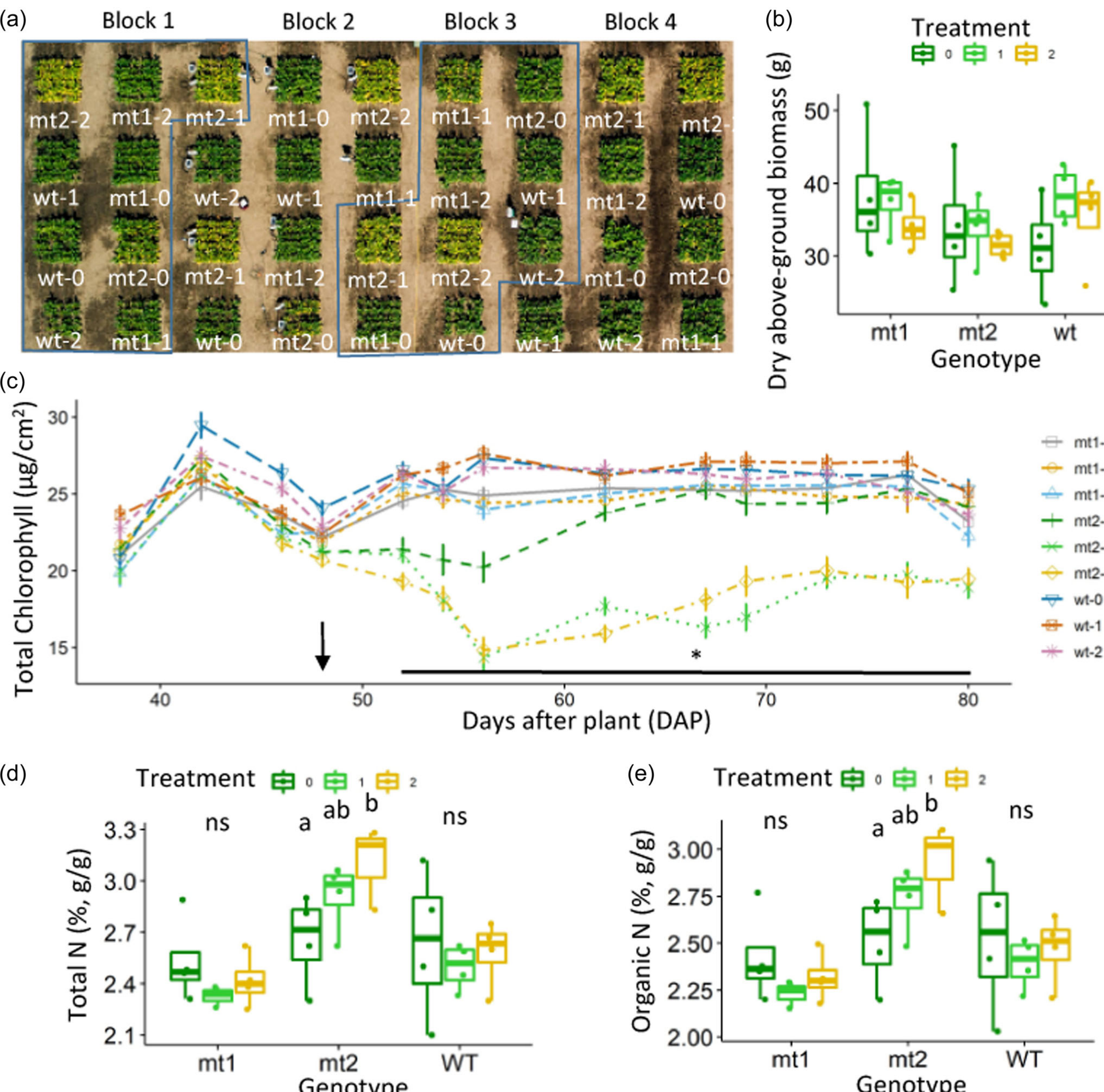

FIGURE 4 Up to 50% chlorophyll reduction after canopy closure results in no change in above-ground biomass in the field tests. (a–c) 2019 Illinois field experiment ($n = 4$ blocks). Three genotypes—wild-type (WT), mutant 1 (mt1), and mutant 2 (mt2)—were treated with three treatments: no spray (−0), 2% ethanol spray once daily (−1), twice daily (−2). (a) Aerial view of the field trial in the 2019 IL field on 30 July 2019 (60 DAP and 12 DAT). (b) Above-ground biomass. (c) Chlorophyll level of sun leaves during field experiment. The black arrow indicates when the spraying started. *Significant differences between treated mt2 and WT when present. (d) Concentration of total nitrogen (%, g/g). (e) Concentration of organic nitrogen (%, g/g). Letters indicate significant differences within genotype when present. The statistical analysis was done using a linear mixed model ANOVA with post hoc Tukey tests ($\alpha = 0.05$). NS, not significant. [Color figure can be viewed at wileyonlinelibrary.com]

3.4 | Reducing the chlorophyll content of leaves did not significantly change the canopy carbon assimilation rate

To assess the impact of reduced chlorophyll levels on leaf and canopy photosynthesis, the absorbed light and CO_2 response of net CO_2 assimilation rate (A_{net}) were measured on sun-exposed leaves in three-field seasons [IL2019, PR2019 and IL2020 (Sun)]. In addition, a shade leaf (four nodes below the sun leaf) was also measured for a single field season [IL2020 (Shade)] to assess gas exchange in lower canopy leaves. Despite the significant reduction in chlorophyll levels observed in all mt2 field trials, we observed

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minimal differences on a leaf area basis between low chlorophyll leaves and full green leaves for all photosynthesis parameters in the light response curves (A/Q_{abs} curves; Figure 5a–d). The respiration rate (R), the light-saturated rate of net CO_2 assimilation (A_{satQ}), and the empirical curvature factor (θ) were not different between low chlorophyll and full green leaves for any of the A/Q_{abs} curve data sets (Table 1). Reductions in the maximum quantum yield of CO_2 assimilation (Φ_{CO2}) were observed in IL2019 and PR2019 experiments for the low chlorophyll but not in IL2020 (Table 1). For the IL2020 field season, the maximal photochemical yield of photosystem II obtained at low light ($\Phi_{PSII(LL)}$) was lower in leaves with low chlorophyll content, but this was not consistently observed in the other field trials (Table S2). The response of A_{net} to internal CO_2 concentration (C_i; A/C_i curves; Figure 5e–h) showed inconsistent differences in photosynthetic parameters, while the empirical curvature factors for the A/C_i curves (ω) were consistently lower in low chlorophyll leaves (Table S2).

The total net carbon assimilation of the canopy (A') was calculated by using a simple three-leaf level concept (Figure S13) simulating an LAI of 3 similar to measurements from the field trial (Figure S8). No difference in canopy photosynthesis between low chlorophyll and dark green canopies (Table 1) was observed for all field trials. We used IL2020 gas exchange data measured from the shade leaf (four nodes below the sun leaf) for the second and third levels in the canopy calculation; IL2020 sun leaf gas exchange data were used for the uppermost leaf layer in the calculation. A' of IL2020 shade data were not significantly different between low chlorophyll and dark green canopies (Table 1). Overall, we did not observe a significant change in canopy photosynthesis between low chlorophyll and dark green controls in any of the three-field experiments.

4 | DISCUSSION

In this study, we explored the benefits of using an inducible promoter to test our hypothesis that the reduction of leaf nitrogen investment in chlorophyll and associated chlorophyll-binding proteins during seed fill could significantly increase seed nitrogen without loss of biomass in both greenhouse and field conditions. Although physically inducible promoters, such as heat (Masclaux et al., 2004) or light (Müller et al., 2014), are perhaps more practical for scalable agriculture than chemically inducible promoters due to environmental issues and operating costs, the ethanol inducible promoter used in this study was more than adequate for proof of concept. We confirmed our prediction in the field and greenhouse that the plant is able to reallocate nitrogen not used in chlorophyll production to increase seed nitrogen. In all trials reported in this study, regardless of season or location, reductions of chlorophyll from 50% to 68% had no effect on biomass relative to neighbouring plants with 100% chlorophyll. We effectively demonstrated that the overproduction of leaf chlorophyll that likely evolved to give these plants a competitive edge in natural environments is not necessary to maintain expected agricultural yields and that those resources can effectively be used elsewhere in seed filling. The work presented in this study confirms model predictions with empirical evidence and sets the groundwork for numerous future research questions concerning the reallocation of nitrogen within the plant.

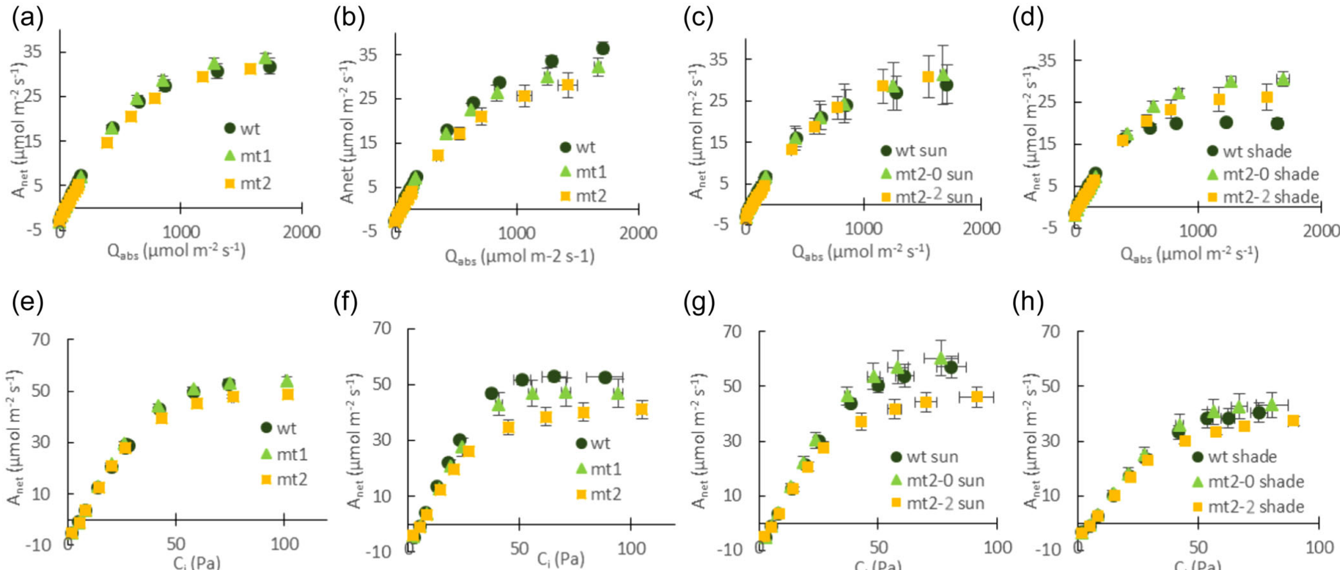

FIGURE 5 Photosynthetic capacity of low chlorophyll and dark green controls from three-field trials. Panels a–d show the light response curves. Panels e–h show the CO_2 response curves. (a, e) IL2019 (n = 4); (b, f) PR2019 (n = 3); (c, g), IL2020 sun leaf (n = 4); (d, h), IL2020 shade leaf (n = 3). IL2019 data (n = 4) and PR2019 (n = 3) compared genotypes within the treatment, 2% ethanol spray twice a day. IL2020 (n = 4) has two genotypes, WT and mt2, which were treated with 2% ethanol spray twice a day, in addition to mt2 without ethanol treatment (mt2-0). Shapes indicate the mean of three to four field plots with ±standard error. [Color figure can be viewed at wileyonlinelibrary.com]

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TABLE 1 Low and high chlorophyll leaf pigment and photosynthetic parameters from three-field trials.

Field	Genotype	*n*	SPAD	Φ_{CO2} (mol/mol)	*R* (μmol m^{-2} s^{-1})	A_{satQ} (μmol m^{-2} s^{-1})	*θ*	A'_{net} (mol m^{-2} day^{-1})
IL2019 (T2)	WT	4	43.6 ± 1.2a	0.059 ± 0.002a	2.46 ± 0.10	38.3 ± 2.5	0.79 ± 0.04	1.15 ± 0.07
	mt1		40.3 ± 2.2a	0.059 ± 0.001a	2.64 ± 0.17	40.7 ± 1.0	0.81 ± 0.02	1.21 ± 0.07
	mt2		26.4 ± 1.7b	0.052 ± 0.002b	2.63 ± 0.23	39.7 ± 1.6	0.78 ± 0.02	1.11 ± 0.03
PR2019	WT	3	45.7 ± 1.6a	0.060 ± 0.003a	2.21 ± 0.22	46.4 ± 2.9	0.67 ± 0.07	1.32 ± 0.02
	mt1		35.7 ± 4.2a	0.064 ± 0.002a	2.66 ± 0.03	41.3 ± 3.4	0.62 ± 0.07	1.15 ± 0.06
	mt2		19.7 ± 4.3b	0.050 ± 0.002b	2.61 ± 0.26	39.3 ± 4.8	0.64 ± 0.05	1.02 ± 0.09
IL2020 (sun)	WT	4	43.7 ± 2.9a	0.056 ± 0.006	2.54 ± 0.25	34.8 ± 4.1	0.73 ± 0.02	0.98 ± 0.11
	mt2-0		38.4 ± 2.4a	0.063 ± 0.007	3.05 ± 0.24	41.5 ± 8.1	0.48 ± 0.13	0.98 ± 0.18
	mt2-2		20.7 ± 1.6b	0.051 ± 0.000	3.12 ± 0.16	40.6 ± 4.9	0.62 ± 0.10	0.96 ± 0.14
IL2020 (shade)	WT	3	33.2 ± 3.6ab	0.058 ± 0.007	1.10 ± 0.15	22.5 ± 1.0a	0.72 ± 0.02	1.17 ± 0.18
	mt2-0		39.5 ± 6.2a	0.053 ± 0.002	1.57 ± 0.19	34.4 ± 2.0b	0.89 ± 0.03	1.18 ± 0.22
	mt2-2		23.2 ± 1.0b	0.052 ± 0.004	1.58 ± 0.13	30.0 ± 4.1b	0.90 ± 0.02	1.23 ± 0.14

4.1 | Developmental stage-specific reduction in leaf chlorophyll increases the concentration of seed nitrogen while maintaining yield and biomass

As chlorophyll biosynthesis and associated pigment binding protein production represent a substantial use of nitrogen resources, an 80% reduction in chlorophyll production could potentially free up to 9% of leaf nitrogen for other uses by the plant that would offer more benefit to agriculture (Walker et al., 2018). We showed that reducing chlorophyll in tobacco leaves in later plant developmental stages, after canopy closure and during seed filling, resulted in increased nitrogen concentration in the seeds while maintaining biomass and seed yields (Figures 2 and 3). Many efforts have been made to maximise nitrogen in the seeds to improve seed quality. The nitrogen content of the seed can be increased when the free amino acid concentrations are increased in the leaf (Caputo et al., 2001) or when the remobilisation of leaf nitrogen is accelerated (Taylor et al., 2010; Zhao et al., 2015). Accelerated leaf senescence increases seed protein content (Uauy et al., 2006), while delayed leaf senescence decreases seed protein content (Zhang et al., 2010). Noticeably, delayed leaf senescence increases seed yield due to continuing photosynthetic activity and carbon fixation (Zhang et al., 2010).

The timing of leaf senescence controls the mobilisation and allocation of carbon and nitrogen resources (Havé et al., 2017). An individual leaf cannot fully support both carbon assimilation and nitrogen remobilisation at the same time, causing the inverse relationship between seed yield and nitrogen content (Havé et al., 2017). Many plants evolved under largely nitrogen-deficient conditions compared to modern agriculture and take up nitrogen whenever it is available to store in Rubisco and other proteins to hoard this normally scarce resource (Denison et al., 2003), even producing more leaves than are required to capture light (Srinivasan et al., 2017). Nitrogen is transferred to seeds only later in development when soil nitrogen is most limiting. Reducing investment in leaves has been proposed to benefit nitrogen use efficiency under modern agriculture conditions that provide sufficient nitrogen (Denison et al., 2003). Supplying nitrogen during seed filling would be beneficial for crop production (Denison et al., 2003); however, using the nitrogen already stored inside the plants would be more economically advantageous than supplying extra fertiliser. Reducing chlorophyll in this study did not initiate senescence, thus, leaves were healthy and maintained photosynthetic activity and carbon fixation (Figure 5; Table 1). The timing of the reallocation of nitrogen resources is critical for the proposed benefit because plants need nitrogen in the leaf for carbon assimilation, so the nitrogen must not be diverted from this purpose too early in development. Lowering the chlorophyll level of the leaf during seed filling is a novel strategy to improve seed quality while maintaining yield.

The effect of reducing chlorophyll on seed nitrogen may vary depending on the availability of nitrogen. In this study, we observed a significant increase in seed nitrogen concentration by 17% under slightly deficient nitrogen conditions (0.8 g urea) in the greenhouse (Figure 2) and a 7% increase under field conditions with sufficient nitrogen supply (Figure 3). We speculate that tobacco plants prioritise the allocation of saved nitrogen from reduced leaf chlorophyll for their survival when nitrogen is scarce (0, 0.2 and 0.4 g urea in Figure 2), while the plants increase the nitrogen concentration in the seeds only when the demand for nitrogen in critical functions has been satisfied. Under sufficient nitrogen conditions in the greenhouse experiment, we noticed a modest 4% increase in seed nitrogen concentration (mean 4.07 vs 3.87 in Figure 2), but the adjusted *p* value ($p = 0.11$) for minimising Type I error rejects the hypothesis, while the hypothesis would be accepted with a raw *p* value ($p = 0.03$). The adjusted *p* value often increases the chance of making Type II errors (Feise, 2002). Perhaps the observed increase under sufficient nitrogen conditions is genuine, as supported by the field experiment. Compared to when sufficient nitrogen is provided, the impact of reduced chlorophyll on seed nitrogen content becomes more

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pronounced when nitrogen availability is slightly limited. This suggests that under slightly limited nitrogen conditions, there may be a greater capacity for the seeds to store saved nitrogen.

Controlling nitrogen remobilisation has a different effect depending on the species. Each species has a different portion of seed nitrogen that comes from the remobilisation of nitrogen stored in roots and shoots before flowering: wheat (*Triticum aestivum* L.) is 60%–95% (Palta & Fillery, 1995); maize (*Zea mays* L.) is 45%–65% (Rajcan & Tollenaar, 1999). Tobacco (cv. Petite Havana), like other plants, also remobilises nitrogen from leaves to sink reproductive tissues when flowering. In this study, the chlorophyll levels of WT and mutants kept increasing right before flowering, then decreased during seed filling and bounced back within 5–6 days (Figures 4c; Figure S11). Demands for nitrogen can stimulate organic nitrogen remobilisation that is mediated by autophagy and vacuolar proteases (Tegeder, 2014). Nitrogen-related macromolecules such as Rubisco and the chlorophyll-binding light-harvesting complex (LHC) are broken down into amino acids, peptides and ureides then transferred to nitrogen-demanding organs by various transporters (Tegeder, 2014). Reduced chlorophyll leads to the degradation of excess LHCs (Tanaka & Tanaka, 2011), and then the organic nitrogen is remobilized to sink tissues such as seeds. The amount of remobilized organic nitrogen can have an impact on seed nitrogen content (Havé et al., 2017). The analysis of nitrogen components in the harvested leaves from a single field season (IL2019), which was not replicated in the other field experiments, revealed an increase in the concentration of organic nitrogen in the leaves of the mt2 genotype with the ethanol treatment (Figure 4e). This suggests that reducing chlorophyll increased the pool size of organic nitrogen in the low chlorophyll leaves, and the plant thus had more nitrogen available to remobilise to the seed during leaf senescence. In addition, the reduced V_{cmax} in the shaded leaves indicates the accelerated breakdown of Rubisco in the shaded leaves of low chlorophyll mutants (Table S3). Further investigations are required to understand the specific protein (likely LHC and perhaps Rubisco) responsible for the increase in organic nitrogen concentration within the low chlorophyll leaves.

4.2 | The benefits and limitations of utilising ethanol inducible gene expression system

Inducible or time-specific gene regulation may open new opportunities for improving nitrogen-use efficiency in crop plants. Most transgenic plant studies have used constitutive promoters; for example, the 35S promoter has been a favourite choice due to its strong expression, often producing a clear phenotype (Borghi, 2010). However, constitutive gene expression of a transgenic trait is not desired in all cases, particularly when targeting essential genes such as *CHLI*. In such cases, inducible promoters can provide advantages (Kizis et al., 2001; Selvaraj et al., 2020). For example, an abscisic acid/stress-inducible promoter was found to regulate genes spatially and temporally, improving root architecture (Chen et al., 2015) and drought tolerance (Selvaraj et al., 2020) without yield penalty. Among 41 studies with the goal of improving nitrogen use efficiency (reviewed by Xu et al., 2012), 32 studies used constitutive promoters to manipulate the expression of genes involved in nitrogen uptake and metabolism. Different timings of fertiliser application have a different effect on yield and seed nitrogen. Yield and seed nitrogen content are highly correlated with nitrogen supply level and availability, particularly at the seed-filling stage (Masclaux-Daubresse & Chardon, 2011). By using the ethanol inducible promoter, we were able to strongly reduce chlorophyll levels once the canopy had closed and plants were producing seeds. Four field trials showed that reducing chlorophyll levels did not cause a penalty to above-ground biomass (Figures 4, S11 and S12), yield (Figures 2 and 3) or canopy photosynthesis rate (Figure 5; Table 1) but did increase seed nitrogen concentration (Figures 2 and 3).

The expression of the ethanol-inducible promoter is dose-dependent for the inducer and sensitive to vapour, which can be used to activate expression (Roslan et al., 2001; Sweetman et al., 2002), limiting the use of the spray method in closed chambers. The response is quite rapid and sustained at a high level; for example, luciferase expression could be detected in the roots of *Arabidopsis thaliana* seedlings within 1 h of ethanol induction (Roslan et al., 2001). The expression peaks between 3 and 5 days after initial induction (Garoosi et al., 2005; Salter et al., 1998) and maintains a high level via subsequent inductions (Roslan et al., 2001; Schaarschmidt et al., 2004). Similarly, the current study showed expression of *CHLI* sRNA within 3 h of ethanol induction, a peak at 3 days, and then a reduction of chlorophyll within 5 days (Figure 1e,f).

The ethanol inducible promoter can be induced unintentionally because ethanol can be produced in plants under certain conditions. The ethanolic fermentation caused by hypoxia/anoxia converts pyruvate to acetaldehyde, which is later converted to ethanol by ethanol dehydrogenase (Drew, 1997). We observed such an unexpected induction in the IL2019 field experiment, presumably by anoxia. Between 55 and 56 DAP, leaves of mt2 in all blocks, including plots without ethanol treatment, turned yellow (see mt2-0 in Figures 4c and S7). After 3 days, chlorophyll production resumed in the untreated mutants. There had been a 0.3-in. rain when the temperature was 15°C at the lowest point and 26°C at the highest point on 52 DAP. It is unclear why only 1 day of moderate rainfall created an anoxic condition out of all four field trials, but this seems to us the most likely explanation for the induction of the RNAi system in the untreated mutants (mt2-0).

We unexpectedly encountered different responses of mt1 to the ethanol application in different environments. We were only able to acquire two mutant events (mt1 and mt2) responding to the ethanol treatment under greenhouse conditions (Figures 1d, S3 and S4). We brought both mutant lines to two field seasons, but mt1 did not respond to the ethanol spray in either field season (Figures 4c, S6, S7 and S11). While both mutants demonstrated robust responses in the greenhouse, their patterns of reaction displayed subtle disparities. Notably, mt2 showed a more rapid and widespread change in leaf colour, while mt1 displayed a slower change and mosaic-like colour

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pattern (Figure S5). Further investigation revealed that the T-DNA insertion in mt1 is located only 1095 bp upstream of the transcription start site of the phosphatidylethanolamine binding protein gene (Nitab4.5_0000161g0080), while the T-DNA insertion in mt2 is located in an intergenic region, with the nearest gene 21.7 kb away (Figures 1c, S1 and S2). Despite these efforts, we can only speculate that the different locations of T-DNA mediate the differential responses of the two mutants to ethanol treatment under field conditions. With these constraints leaving us only one mutant to analyse, we compared the treated and untreated mt2 plants, suggesting that the observed results under field conditions were unlikely due to the positional effects.

4.3 | Potential benefits of reducing chlorophyll levels in crops

The potential benefits of reducing chlorophyll levels in crops for mitigating further warming have been proposed through reducing radiative forcing due to the higher albedo of low chlorophyll crops (Cutolo et al., 2023; Genesio et al., 2021; Lugato et al., 2020). It is tempting to propose that a reduction in chlorophyll production can maintain the quality of seeds in future climate environments where the elevated CO_2 concentration accelerates carbon assimilation and increases seed carbon-to-nitrogen ratios in nonleguminous species (Myers et al., 2014). Increased carbon assimilation changes the stoichiometric balance of nutrients in plants by increasing carboxylation but not the absorption of minerals (Loladze, 2002). A meta-analysis of free-air carbon dioxide enrichment (FACE) experiments showed that wheat seed protein was reduced by 6% and rice seed protein by 8% under the elevated CO_2 conditions that also led to increased photosynthesis (Myers et al., 2014), which would disturb the carbon to nitrogen (C/N) balance by producing more carbon (McGrath & Lobell, 2013). Our work here demonstrates that reducing chlorophyll by about 60% in tobacco leaves increases the nitrogen concentration of the seeds by up to 17%, depending upon the amount of nitrogen fertilisation (Figures 2 and 3). Therefore, reducing chlorophyll could maintain the C/N ratio by increasing nitrogen to match the increased carbon from improved photosynthesis. The balance of these two nutrients, markers of seed quality, could be maintained with no penalty on biomass or seed production using this method.

This study did not show improvement in canopy photosynthesis in low chlorophyll mutants (Figure 5; Table 1), consistent with some studies with low chlorophyll soybean mutants (Slattery et al., 2017; Walker et al., 2018) but contrary to the model and empirical studies (Gu et al., 2017; Kirst et al., 2017; Li et al., 2013; Ort et al., 2011; Sakowska et al., 2018; Song et al., 2017; Walker et al., 2018). The decrease in chlorophyll levels may down-regulate not only LHC but also other photosynthetic apparatus, potentially leading to a reduced photosynthetic capacity (Tanaka & Tanaka, 2011). Our results indicate that the maximum rate of electron transport (J_{max}) is significantly lower in IL2019 and IL2020, though this was not seen in PR2019 (Table S3). This suggests that saved nitrogen from reducing chlorophyll and LHC may be redirected toward seed nitrogen rather than enhancing the photosynthetic capacity through rate-limiting enzymes. We observed an increase in organic nitrogen (Figures 4d,e, S9 and S10), but the specific protein responsible for this increase was not investigated. A modelling study projected a 30% increase in canopy photosynthesis if the reduction in leaf chlorophyll levels decreased LHC specifically while maintaining total nitrogen in other photosynthetic apparatus (Song et al., 2017). Perhaps, further interventions would be required to redirect nitrogen savings from reduced chlorophyll-LHC to the most beneficial targets for increasing photosynthesis (Song et al., 2017). Other considerations proposed include maintaining an optimal chlorophyll a/b ratio (Friedland et al., 2019) and photosystem II efficiency (Mao et al., 2023), as well as the timing and degree of regulation on target genes with minimal pleiotropic effects (Slattery & Ort, 2021) to improve canopy photosynthesis by optimising chlorophyll levels.

In summary, to understand the benefits of reducing chlorophyll in later developmental stages, we created an inducible RNAi tobacco mutant that expressed *CHLI* sRNA within 3 h of induction and reduced chlorophyll within 5 days in field conditions. Once the plants had grown enough and the canopy closed, we reduced chlorophyll levels by up to 68% without penalty to above-ground biomass or canopy photosynthesis. Chlorophyll reduction of the leaf in the seed-filling stages increased nitrogen concentration in the seeds, while biomass and seed yields were maintained. On top of the breeding efforts to maximise yield and seed quality, we suggest the time-specific reduction of chlorophyll as a novel strategy to decouple the inverse relationship between yield and seed nitrogen.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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