



A Versatile High Throughput Screening Platform for Plant Metabolic Engineering Highlights the Major Role of *ABI3* in Lipid Metabolism Regulation

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Traditional functional genetic studies in crops are time consuming, complicated and cannot be readily scaled up. The reason is that mutant or transformed crops need to be generated to study the effect of gene modifications on specific traits of interest. However, many crop species have a complex genome and a long generation time. As a result, it usually takes several months to over a year to obtain desired mutants or transgenic plants, which represents a significant bottleneck in the development of new crop varieties. To overcome this major issue, we are currently establishing a versatile plant genetic screening platform, amenable to high throughput screening in almost any crop species, with a unique workflow. This platform combines protoplast transformation and fluorescence activated cell sorting. Here we show that tobacco protoplasts can accumulate high levels of lipid if transiently transformed with genes involved in lipid biosynthesis and can be sorted based on lipid content. Hence, protoplasts can be used as a predictive tool for plant lipid engineering. Using this newly established strategy, we demonstrate the major role of *ABI3* in plant lipid accumulation. We anticipate that this workflow can be applied to numerous highly valuable metabolic traits other than storage lipid accumulation. This new strategy represents a significant step toward screening complex genetic libraries, in a single experiment and in a matter of days, as opposed to years by conventional means.

Keywords: high throughput screening, protoplast, metabolic engineering, lipid accumulation, fluorescence activated cell sorting

INTRODUCTION

Modern societies have slowly transitioned, over the past couple of centuries, from a bio-based economy to a petrol-based economy. Agriculture consequently transitioned to a food/feed focus whereas it used to meet all demands for energy and materials too. Driven by the threat of climate change and a need for more sustainability, re-establishing a bio-based economy is currently a

major global challenge. To compete with established petrochemical industries, crops need to be re-engineered to have new traits or produce new compounds. However, current crop metabolic engineering approaches are slow and difficult, and this creates a significant bottleneck in the development of new crop varieties that are tailored to meet future agricultural needs in an ever changing environment.

As reflected by the late Richard Feynman's statement: "What I cannot create, I do not understand," the major challenge in crop metabolic engineering is to find the right set of genetic components necessary to obtain a desired trait. This is particularly due to traditional functional genetic studies in crops being time consuming, complicated and not usually compatible with high throughput. Indeed, it usually takes several months to over a year to obtain desired mutants or transgenic plants (Ishida et al., 2007; Yadava et al., 2017) necessary to study the effect of single genetic components on specific traits of interest. The testing of complex metabolic or genetic circuits thus seems like a herculean task. The use of model plants, such as *Arabidopsis thaliana*, with fully annotated genomes and much faster and cheaper gene delivery systems (Clough and Bent, 1998), had an undeniable impact on crop improvement (Provar et al., 2016), but still has limitations regarding time, scale and technology transfer between species.

While more promising, few transient transformation systems have been developed to test genetic components for crop re-engineering strategies. Currently, the most widely used transient transformation system for rapid gene testing is agroinfiltration of *Nicotiana benthamiana* leaves (Yang et al., 2000), which allows for the delivery of one or multiple genetic components to test multigene circuits in a few days (Wood et al., 2009). Pioneering studies have already demonstrated the power of such efficient transient transformation systems. For example, the "Leaf Oil" platform technology, which allows plants to accumulate oil in all vegetative organs (Vanhercke et al., 2017) with yields potentially exceeding those of canola and oil palm, was quickly developed thanks to an extensive number of design-built-test-learn cycles (Vanhercke et al., 2013, 2014; El Tahchy et al., 2017) that strongly relied on an efficient use of the agroinfiltration-based system. The technology is continuously being upgraded by further cycles to create tailor-made products for biofuel applications (Reynolds et al., 2015; Reynolds et al., 2017), and is also being transferred to a range of major crops such as sugarcane (Zale et al., 2016), maize (Alameldin et al., 2017) and *Sorghum bicolor* (Vanhercke et al., 2019). Such high biomass crops have the potential to become a future sustainable and abundant supply of plant oils for food, feed, biofuel and oleo-chemical applications (Rahman et al., 2016; Weselake, 2016). This type of assay is not limited to studying leaf specific metabolic pathways. Petrie et al. (2010) demonstrated that co-infiltration of the tested components in combination with the *A. thaliana* *LEAFY COTYLEDON2* (*LEC2*) gene results in a leaf with a seed-like gene regulation profile, thus allowing for the testing of seed-specific components. A modified version of this assay was also developed in transgenic *LEC2* induced *Brassica napus* somatic embryos for testing seed-specific components with increased reliability to predict seed traits (Belide et al., 2013). These assays allowed for the rapid gene testing and assembly

of the $\omega 3$ fish-like oil synthetic seed biosynthesis pathway that was first deployed in *A. thaliana* as proof of concept (Petrie et al., 2012), and then in oilseed crops such as *Camelina sativa* (Petrie et al., 2014) and canola (Walsh et al., 2016), with $\omega 3$ production at industrially relevant levels. Nevertheless, these transient transformation systems are still limited in terms of scale, species and tissue specificity, and other transient assays are required to overcome these limitations.

Another type of transient system based on protoplast transfection has been available for more than 50 years, and now represents a very promising system for rapid testing of genetic components in most plants, and for several reasons. First, protoplasts can be isolated from almost any tissue of any crop (Burris et al., 2016), thus allowing better predictability through more species-targeted approaches than the agroinfiltration-based system that is established only in tobacco and few other species. Second, protoplasts are single cells, thus allowing more precise studies than multicellular systems to address tissue and cell-type specific questions. Thirdly, a very large number of protoplasts can be isolated from one preparation, allowing testing of many variables simultaneously in one assay. Finally, protoplast transformation is now well established (Yoo et al., 2007), with a higher efficiency than any other plant transformation system (Jiang et al., 2013), and is therefore very well suited for high throughput platforms. The use of protoplast-based systems also allows access to other types of high throughput approaches, such as flow cytometry and cell sorting, which open a wide new range of screening possibilities. Protoplasts have long been extensively used with fluorescence activated cell sorting for several different applications. Such methods were developed from the early 1980s for applications such as protoplast viral infection level monitoring (van Klaveren et al., 1983) and chlorophyll content and size distribution characterization (Galbraith et al., 1988). More recently, similar approaches have been used for more complex studies such as protein accumulation detection (Cronjé et al., 2004), overexpression analyses (Bargmann and Birnbaum, 2009), sorting of particular cell types for RNA isolation and transcriptome-wide analyses (Birnbaum et al., 2005; Bargmann and Birnbaum, 2010), or to study cell-type specific expression during both development and stress (Grønlund et al., 2012). Such techniques allow for the direct screening of millions of variants in a very short amount of time, and ongoing improvements (You et al., 2014) allow for the development of increasingly powerful high throughput screening methods. For example, recently, cell sorting based on the lipid content of thousands of mutant lines of a green unicellular algae was achieved in a few hours, followed by a few additional days for cellular multiplication and additional lipid analyses to confirm the phenotype (Terashima et al., 2015), representing one of the most powerful mutant screens ever reported for green vegetal cells. While random mutagenesis is not available for protoplasts, transient transformation is very efficient, and transformation of expression libraries could be carried out to generate large numbers of transformed cells for rapid trait evaluation by flow cytometry. However, sorting of plant protoplasts based on a high value trait, such as lipid accumulation, has not yet been reported.

The regulation of lipid biosynthesis and storage in plants has been extensively studied. The highly conserved plant transcription factors ABCISIC ACID INSENSITIVE 3 (*ABI3*) (Giraudat et al., 1992), *FUSCA3* (*FUS3*) (Luerssen et al., 1998), *LEAFY COTYLEDON1* (*LEC1*) (Meinke et al., 1994; West et al., 1994), and *LEC2* (Chen et al., 1997) are the major master regulators that control the gene regulation networks governing most seed developmental mechanisms (Suzuki et al., 2003; Braybrook et al., 2006; To et al., 2006; North et al., 2010; Wang and Perry, 2013). The *abi3*, *fus3*, *lec1*, and *lec2* mutants share common phenotypes such as reduced storage protein accumulation and anthocyanin accumulation (To et al., 2006). *FUS3*, *LEC1*, and *LEC2* also have been reported to trigger triacylglycerol accumulation in seeds, leaves and liquid cell culture (Lotan et al., 1998; Zhang et al., 2002, 2016; Santos Mendoza et al., 2005; Tan et al., 2011; Vanhercke et al., 2017). The major action of *LEC1*, *LEC2*, and *FUS3* on lipid accumulation is mostly through regulating directly or indirectly the expression of the Wrinkled1 (*WRI1*) transcription factor (Casson and Lindsey, 2006; Baud et al., 2007; Santos-Mendoza et al., 2008; Yamamoto et al., 2010; Wang and Perry, 2013; Marchive et al., 2014), but the complete picture of *WRI1* regulation is still unclear (Kim et al., 2016). *WRI1* function is highly conserved amongst plants. *WRI1* is a node acting at the interplay between these master regulatory elements and fatty acid metabolism. *WRI1* directly regulates the transcription level of many genes essential for fatty acid synthesis to trigger seed oil production (Baud et al., 2007; Pouvreau et al., 2011). After it was demonstrated that *WRI1* overexpression increases oil accumulation without undesirable side effects (Shen et al., 2010; Pouvreau et al., 2011), *WRI1* became a major target of metabolic engineering approaches to increase oil content (Vanhercke et al., 2013, 2014, 2017). By contrast, even though many of *ABI3* targeted genes are known to function in seed oil storage (Wang et al., 2007; Monke et al., 2012), to date, little is known about the direct effect of *ABI3* on lipid content in plants.

In this study, we demonstrate that protoplasts from 15-day-old tobacco leaf can accumulate high levels of lipid if transiently transformed with genes involved in lipid biosynthesis. We observed a direct correlation between protoplast lipid content and protoplast fluorescence intensity after lipid-specific fluorescent staining. Furthermore, we show that protoplasts can be sorted based on lipid content and further used for downstream processing and analyses. Transient gene expression in protoplasts produced metabolic results similar to those reported for stably transformed plants, in terms of lipid accumulation. We demonstrate that combinatorial gene testing is possible in protoplasts and we observed a similar synergistic effect of the genes *WRI1* and *DGAT1* on increasing lipid accumulation in protoplasts, compared with previously published studies conducted in transiently and stably transformed leaves (Vanhercke et al., 2013, 2014). Taken together, our results demonstrate that tobacco leaf protoplasts are a reliable model for plant lipid engineering. This platform constitutes a new high throughput tool to develop plant lipid engineering strategies, but also an alternative strategy to study plant lipid metabolism in general, such as the relative effects of major

known elicitors of lipid accumulation in plants. We compared the effect of *ABI3*, *FUS3*, *LEC1*, and *LEC2* on lipid accumulation in protoplasts. Our results confirm that *ABI3* is an important regulator of oil accumulation and demonstrate for the first time the direct correlation between *ABI3* overexpression and increased lipid content in plants. These results also suggest that *ABI3* might trigger lipid accumulation partly through pathways independent from *WRI1* and *LEC2* regulation.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Plants used in this study all originated from the *Nicotiana tabacum* cultivar Wisconsin 38, including wild-type (WT) and a T4 generation *WRI1-DGAT1-OLEOSIN* transgenic line, referred to as High Oil (HO), for the high levels of triglycerides accumulating in its leaves (Vanhercke et al., 2014). Seeds were surface-sterilized by 2 min incubation in 70% ethanol, followed by 10 min incubation in 1.8% bleach and five washes in sterile water, all with frequent agitation. Seeds were sown on half MS media, supplemented with 3% sucrose, in petri dishes and placed in the dark at 4°C for 4 days. Plants were grown in a growth cabinet under 16 h day/8 h night light conditions (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at 22°C/18°C, respectively.

Plasmids DNA Construction and Preparation

Vectors were designed as minimal plant expression constructs for production in large quantity in *Escherichia coli* and strong transient expression in tobacco protoplasts. A modified pENTR11 vector (Invitrogen) was used as a minimal backbone after removal of the *ccdB* operon, conserving only kanamycin resistance and the *E. coli* origin of replication. The chemically synthesized (GeneArt) *CaMV35S* promoter, coding sequence (*A. thaliana* *WRI1*, *DGAT1*, *ABI3*, *FUS3*, *LEC1*, or *LEC2*, respectively AT3G54320.1, AT2G19450.1, AT3G24650.1, AT3G26790.1, AT1G21970.1, or AT1G28300, codon optimized for tobacco) and NOS terminator were ligated in this minimal vector. For protoplast transient transformation, plasmid DNA was prepared using the NucleoBond Xtra Midi Kit (MACHEREY-NAGEL, Germany) according to the manufacturer's recommended protocol, and resuspended in ultrapure DNase/RNase-free distilled water (Invitrogen) at 1 $\mu\text{g}/\mu\text{l}$.

Protoplasts Preparation, Transient Transformation and Staining

The whole procedure was conducted under sterile conditions. Plants were collected 15 days after germination. Leaf tissues were harvested and thinly sliced in protoplast buffer (400 mM mannitol, 10 mM MES hydrate, 20 mM KCl, 20 mM CaCl_2 , 2 mM MgCl_2 , pH 5.7, 0.1% bovine serum albumin). Leaf strips were rinsed with fresh protoplast buffer, placed in enzyme solution (protoplast buffer supplemented with 1.2% cellulase Onozuka

R-10, 0.6% cellulase Onozuka RS, and 0.4% macerozyme R-10), subjected to vacuum for 10 min and incubated in enzyme solution for 16 h in the dark at 22°C. After incubation, protoplasts were released with 5 min agitation on a rotating plate at 50 rpm, filtered through a 40 µm cell strainer, washed twice with fresh protoplast buffer, counted on a hemocytometer, and then resuspended in fresh protoplast buffer at 0.25 M cells per ml. For transformation, protoplast samples were incubated for 1 h on ice, the buffer was then removed, pelleted protoplasts were resuspended in an identical volume of MMg solution (0.4 M mannitol, 15 mM MgCl₂, 4 mM MES, pH 5.6), supplemented with 25 µg/ml of each plasmid and then mixed with an identical volume of PEG solution (40% PEG4000, 0.2 M mannitol, 300 mM CaCl₂). After 5 min incubation, protoplasts were washed twice with fresh protoplast buffer, resuspended in fresh protoplast buffer at 0.25 M cells per ml, placed in culture plates and incubated in a growth cabinet under constant light conditions (150 µmol m⁻² s⁻¹) at 22°C. For lipid staining, protoplasts in protoplast buffer at 0.25 M cells per ml were supplemented with 1 µg/ml BODIPYTM 493/503, incubated for 15 min at room temperature in the dark, spun down (300 × g for 5 min) and resuspended in the same volume of fresh protoplast buffer.

Lipid Extractions and Analyses

For total lipid extractions, fresh leaves were cut as for protoplast isolation and freeze-dried whereas freshly isolated protoplasts were spun down (for 5 min) to remove all buffer and then snap-frozen in liquid nitrogen. Samples were ground with a ball bearing in TissuLyser II (QIAGEN, Germany) and homogenized in 36% methanol, 36% chloroform, 20 mM acetic acid, 1 mM EDTA. Total lipid fraction (lower phase) was separated by 2 min centrifugation at 4000 × g, dried under nitrogen and resuspended in chloroform. Total lipid extracts were fractionated by thin layer chromatography fractionation as previously described (El Tahchy et al., 2017), followed by fatty acid methyl ester quantification and analysis by gas chromatography, also conducted as previously described (Vanhercke et al., 2013; El Tahchy et al., 2017). Unless otherwise stated, analyses were conducted on a minimum of three individual samples for each genotype, condition or cell population.

Cell Imaging by Microscopy

Glass slides were prepared by placing four drops of petroleum jelly in a square arrangement and 50 µl of protoplasts at 1 M cells per ml in protoplast buffer within that square. A coverslip was then gently pressed down on the solution and jelly drops. Protoplasts were captured (**Figure 3A**) using a 40× objective on a Zeiss Axio Imager M1 fluorescence compound microscope fitted with an AxioCam HR Rev3 camera (Carl Zeiss Microscopy GmbH, Germany), with bright field and no filter or with UV light and a Filter Set 38 HE (Excitation filter BP 450–490 nm, Beam splitter FT 495 HE, emission BP 500–550 nm) to visualize lipid bodies stained with BODIPYTM 493/503. Images were acquired using the Zeiss Zen imaging software (blue edition, Carl Zeiss Microscopy GmbH, Germany).

Protoplast Flow Cytometry and Fluorescence Activated Cell Sorting

Cell sorting was performed on a BD FACS Aria III (BDIS, San Jose) with a 100 µm nozzle and data were acquired and analyzed using the DIVA 8.0 software. Protoplasts suspended in the protoplast buffer at a concentration of 1 million/ml were acquired at an approximate flow rate of 10 µl/min with mild agitation. Data were acquired for 30,000 particles and after exclusion of doublets, the samples were analyzed for BODIPYTM 493/503 (Excitation 488 nm, Emission 530/30 nm) and chlorophyll auto fluorescence (Excitation 640 nm, Emission 670/30 nm). Cells were sorted based on BODIPY/chlorophyll signal ratio. For each gated population, three independent samples of 0.25 M cells were collected in protoplast buffer.

Flow cytometric analyses were conducted on an Invitrogen Attune NxT Flow Cytometer and analyzed with Invitrogen Attune NxT Software. Cells suspended in the protoplast buffer at a concentration of 0.25 M cells per ml were analyzed at a flow rate of 250 µl/min and data were acquired for 300 µl. BODIPYTM 493/503 fluorescence was excited with a 488-nm laser and emission was captured by a 530/30-nm bandpass filter. Unless otherwise stated, analyses were conducted on a minimum of three individual samples for each condition.

Statistical Analysis

For the statistical analysis presented in **Figure 5**, data were analyzed via a linear mixed model fitted using the “lmer” linear mixed modeling function from the “lme4” package (Bates et al., 2015) of the R statistical software system (R Core Team., 2014). This allowed the analysis to properly account for the impacts of the grouping of processed samples together on plates (via a factor “Plate”), and also recognize that the full dataset is formed from two individual repeats of the same experiment (via a factor “Experiment”). Hence, the model incorporated a (random effects) “blocking” structure consisting of “Sample”, nested within Plate, nested within “Experiment”. Treatment (fixed effects) structure was modeled as a two-way factorial, *WR11* crossed with *DGAT1*, where factors *WR11* (having levels *WR11+* and *WR11-*) and *DGAT1* (levels *DGAT1+* and *DGAT1-*) represent the presence/absence of the corresponding genes in the samples. Hence, the WT treatment corresponds to the combination “*WR11-*:*DGAT1-*” and the samples transformed with *DGAT1*, *WR11*, or both, correspond to “*WR11-*:*DGAT1+*,” “*WR11+*:*DGAT1-*” and “*WR11+*:*DGAT1+*,” respectively. The model allows to test for the “main effect” of *WR11* (the average effect of *WR11* status, regardless of *DGAT1* status), the main effect of *DGAT1* (the average effect of *DGAT1* status, regardless of *WR11* status), and the interaction effect between *WR11* and *DGAT1* (the combined effects of *WR11* and *DGAT1* after having removed effects of the two main effects terms).

In fitting any statistical model, it is important to assess various residual diagnostic plots to ensure that the assumptions underlying the model are met and that predictions and tests derived from the model are reliable. Residual diagnostic plots from the fit of the model to the raw data suggested that the random scatter in the data increases as the mean of the data

increases; a common property of such assay data. To model the data appropriately, a log₁₀ (log to base 10) transformation of the data was required to ensure that the model assumption of constant variance was met. The statistical model was therefore applied to the log₁₀ transformed data, and predicted means were back-transformed as required to obtain predictions on the original data scale. Treatment predicted means and associated statistics were obtained using the R “predictmeans” package (Luo et al., 2018). Tests for the statistical significance of terms in the factorial treatment structure were obtained using the R “lmerTest” (Kuznetsova et al., 2017) package.

The errors bars and *P*-values presented in **Figures 2, 3, 4 and 6** correspond to the standard deviation and a two-tailed t-test with unequal variance.

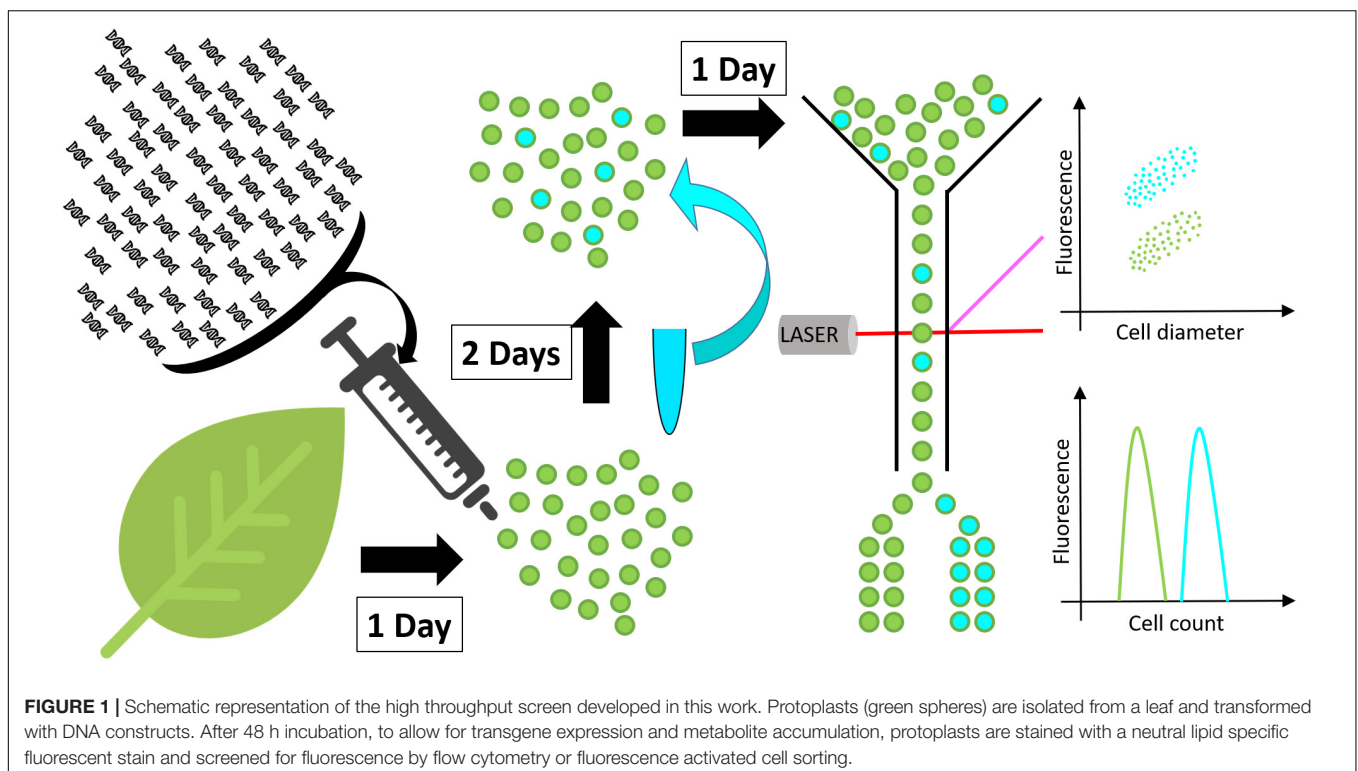
RESULTS

Protoplasts Can Be Used as a Predictive Tool for Plant Lipid Engineering

To confirm that protoplasts can be used as a predictive tool for plant lipid engineering, we tested if the HO phenotype of a previously established stably transformed line could be reproduced with protoplasts isolated from the same line. The surprising lack of studies regarding lipid accumulation in protoplasts to date suggested that such approaches might not be possible to be conducted. We previously reported the establishment of a HO line of tobacco (henceforth referred to as HO) accumulating in its leaves up to 24 times more total lipid, and particularly 76 times more triglycerides, at

maturity, compared to WT (Vanhercke et al., 2014). We also reported that this increased lipid accumulation was gradual during development, thus potentially making it difficult to measure the increase in very young tissue. Leaf tissues from 15 days old WT and HO plants were harvested and partly used for protoplast isolation. The remaining leaf tissues as well as protoplasts isolated from the same leaf samples were then analyzed for their triglycerides and total lipid content (**Figure 2**). The analysis revealed that at this early developmental stage and under the specific growth conditions of this assay, HO plantlets had accumulated about two times more total lipid and almost 90 times more triglycerides than WT, while HO protoplasts had accumulated about 25% more total lipid and more than two times more triglycerides than WT. Thus, despite some variation in intensity, the analysis confirmed that the increase can still be measured at the protoplast level. We also confirmed that even though the variation between the HO line and WT would be easier to measure by focusing on the triglycerides level, it is still quite feasible to measure it at the total lipid level. This will allow for the use of generalist neutral lipid stains, which stain any neutral lipid and not only triglycerides, to measure the variation in oil content.

We can observe a disparity at 15 days between the lipid contents of fresh tissues and protoplasts, indicating that the variation between HO and WT is reduced in the protoplast samples. One reason could be that fresh tissue samples are mostly constituted of fibres, which contains no lipid, and are not present in the protoplast sample. Another explanation is that the total leaf sample contains multiple types of cells, whereas protoplasts isolation methods are notorious to mostly enrich mesophyll cells.



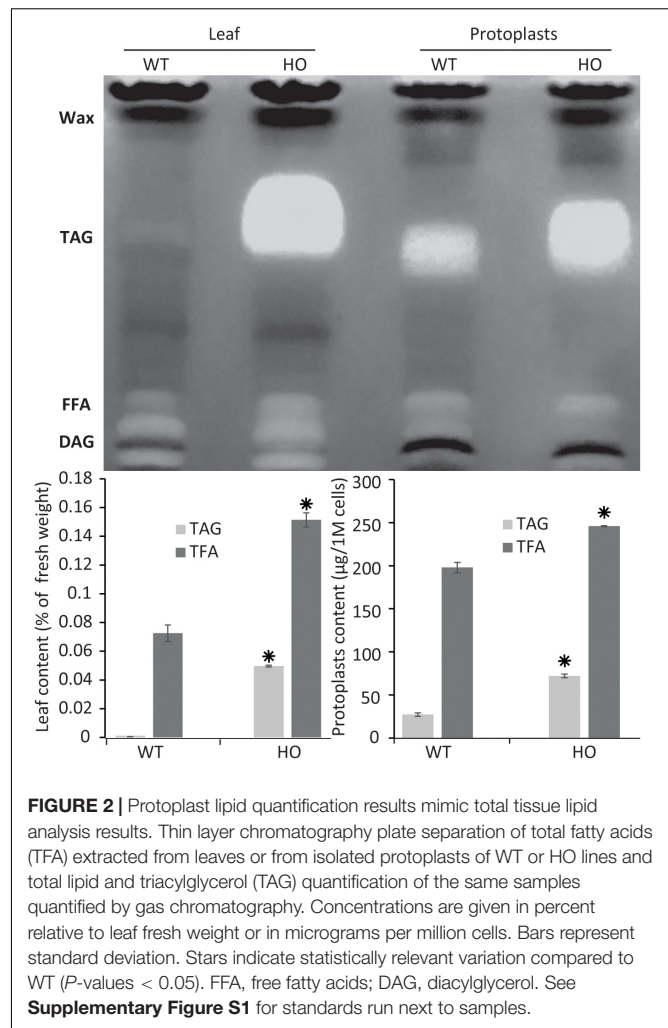
It could be that mesophyll cell contains more TAG than other types of leaf cells, but this was not tested in this study. This could explain why the WT protoplast sample appears to have a richer lipid composition than the fresh tissue it was prepared from, because it concentrates a certain type of cells only. Finally, a recent study demonstrated that the protoplast isolation itself could trigger the accumulation of some lipid species (Barnes et al., 2019), which could also explain why the WT protoplast sample appears to have a richer lipid composition than the WT leaf tissue sample.

A New and Rapid Method for Testing DNA Constructs for Plant Lipid Engineering

The new protoplast-based platform for high throughput plant lipid engineering presented in this study allows for the testing of DNA constructs in regard to their ability to induce lipid accumulation in plant cells within only 72 h. Using BODIPYTM 493/503, a fluorescent neutral lipid stain, lipid quantity in protoplasts is evaluated by measuring cell fluorescence by flow cytometry or alternatively by fluorescence activated cell sorting if specific cell populations need to be separated for further analyses (Figure 1). Protoplasts with high lipid accumulation consequently exhibit higher fluorescence and can be identified and eventually sorted based on the relative intensity of the fluorescent stain per cell.

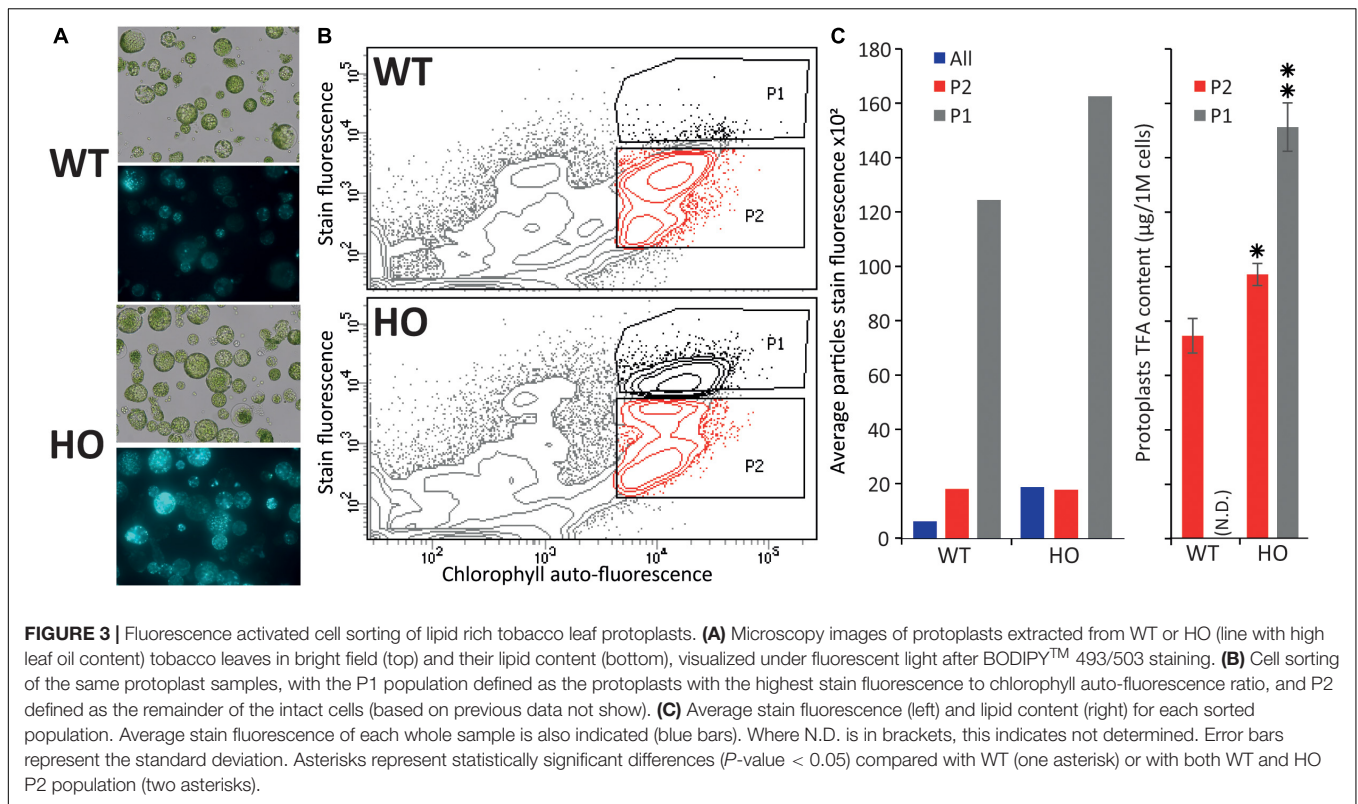
Tobacco Leaf Protoplasts Can Be Sorted Based on Lipid Content

In the previous part, we demonstrated that we can measure variation in protoplast oil accumulation by conducting the analysis at the total lipid level instead of focusing on triglyceride accumulation only, which should allow for the use of a generalist neutral lipid stain, which stains triglycerides and all other neutral lipid, to measure the variation in oil content. We next sought to confirm that protoplasts can be sorted based on storage lipid content. Protoplasts were isolated from the leaves of 15-day-old WT and HO plantlets as described above, and stained with BODIPYTM 493/503, a generalist neutral lipid stain, prior to microscopy (Figure 3A) or flow cytometric analysis (Figure 3B). WT and HO stained protoplasts were consecutively sorted, based on their intensity of BODIPYTM 493/503 fluorescence relative to chlorophyll auto-fluorescence. Previous iterations of sorting coupled with microscopy allowed us to determine where the intact green cells are situated in the graph generated during cell sorting (data not shown). Two distinct populations of intact green cells were gated, with P1 defined as the most intensively stained cells and P2 as the rest of the intact green cells (Figure 3B). Average stain fluorescence per cell was calculated for each population (Figure 3C left). For each population, three samples of 250,000 cells were collected and then subjected to the same total lipid analysis as described above (Figure 3C right). This was not done for the WT-P1 population because it was not possible to retrieve enough cells to conduct the analysis. Several conclusions could be drawn for this assay.



First, we confirmed that BODIPYTM 493/503 staining allows for measuring of the oil content difference that we previously measured between WT and HO protoplasts. The whole HO sample had a higher average protoplast stain fluorescence intensity than the whole WT sample (blue bars in Figure 3C left). This correlates with the results presented in the previous part demonstrating that HO protoplasts accumulate more total lipid than WT and illustrates the direct correlation between lipid content and BODIPYTM 493/503 fluorescence intensity.

Secondly, the flow cytometric analysis showed that the HO sample had a distinct P1 population that segregated from the rest of the cells, while the WT cells attributed to the P1 population corresponded to the top cells of the P2 population rather than being a distinct cell population (Figure 3B). For both WT and HO samples, cells of the P1 population exhibited higher average stain fluorescence intensity compared to cells of the P2 population (Figure 3C left). Lipid analysis revealed that the HO-P2 population was significantly more lipid-rich than the WT-P2 population and that the HO-P1 population was significantly more lipid-rich than both the HO-P2 and WT-P2 populations (Figure 3C right). The variation in total lipid quantified in the



HO-P2 and WT-P2 populations, while these two populations present a very similar level of fluorescence can be explained by the strong disparity between the ratios of different lipid species present in WT and in HO samples (Supplementary Table S1). It is possible that BODIPY™ 493/503 has different affinity depending on lipid species, which was not investigated in this study but could explain why the HO-P2 and WT-P2 populations present an identical fluorescence intensity after staining, while containing a slightly different amount of total lipid. Additionally, BODIPY™ 493/503, while being the most appropriate lipophilic dye available for this study, only binds to neutral lipid, while the total lipid quantification would quantify a much larger spectra of lipid species.

Overall, this assay confirmed that protoplasts can be sorted based on lipid content and that measuring the whole sample BODIPY™ 493/503 fluorescence signal (green bars in Figure 3C left) is enough to evaluate the effect of a gene combination on the overall lipid content in protoplasts. We also note that the variation measured between the WT and HO sample is amplified when using BODIPY™ 493/503 staining coupled with flow cytometry (compared with lipid analysis methods), suggesting that this alternative screening strategy could be more powerful than traditional ones.

Transiently Transformed Tobacco Leaf Protoplasts Can Accumulate Lipid

We have established that leaf protoplasts from a stable transgenic line that accumulates more oil in its leaves than WT also

accumulates on average more oil per protoplast compared to WT and that the difference can be measured by traditional lipid analysis or measured by flow cytometry after protoplast isolation and appropriate staining. However, to date, it was unclear if and how leaf protoplasts would produce an increased quantity of oil compared to WT, after isolation and transient transformation, reflected by the absence of any similar study in the literature.

The stable transgenic line used in the study over-expresses a combination of genes coding for the *A. thaliana* WR1 transcription factor, a regulator of the late glycolysis and *de novo* fatty acid biosynthesis pathways, the *A. thaliana* DGAT1 acyl-CoA: diacylglycerol acyltransferase, a major lipid metabolism enzyme, and an OLEOSIN oil body protein from *Sesamum indicum*, which coats oil droplets and protects them from degradation. The reasoning for including OLEOSIN was that achieving greater oil accumulation in leaves in a long gene-expression-window (stable expression) might have required reducing oil degradation in this non-seed tissue. However, we previously demonstrated that the combined transient overexpression of WR1 and DGAT1 only in *N. benthamiana* leaves was enough to produce a measurable and significant synergistic effect on leaf oil content (Vanhercke et al., 2013).

Based on the results presented above, next we tested transient transformation of leaf protoplasts with genetic constructs carrying expression cassettes for WR1 or DGAT1 or both and measured the difference in protoplast total neutral lipid content, using BODIPY™ 493/503 staining and flow cytometry recording

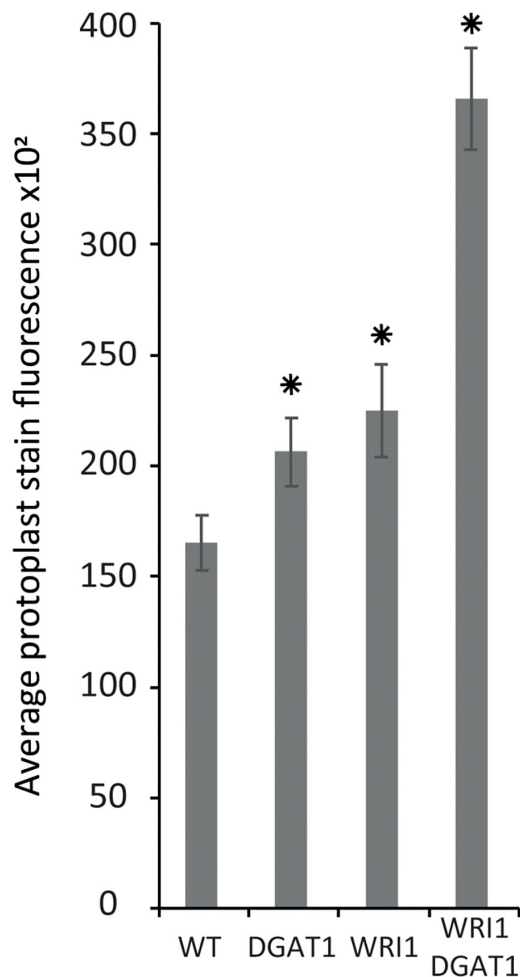


FIGURE 4 | Lipid accumulation in tobacco leaf transiently transformed protoplasts. Lipid accumulation in WT tobacco leaf protoplasts compared to WT protoplasts transiently transformed with *WRI1* or *DGAT1* or both, was measured with BODIPYTM staining and flow cytometry recording of the average particle stain fluorescence for the whole sample. Error bars represent the standard deviation. Asterisks represent statistically significant differences (P -value < 0.05) compared with WT.

of the average protoplast stain fluorescence for the whole sample (Figure 4). Compared to the non-transformed control, leaf protoplasts transiently transformed with *WRI1* or *DGAT1* or both exhibited increased average stain fluorescence, indicating an increase in total neutral lipid content.

We also observed that when co-transformed, the combined effect of *WRI1* and *DGAT1* on lipid accumulation is greater than the additive effect of each individual transformations, illustrating a synergistic effect of *WRI1* and *DGAT1* (Figure 4) toward lipid accumulation in *N. tabacum* leaf protoplast, consistent with previous reports from *N. benthamiana* leaf transient assay (Vanhercke et al., 2013) and stably transformed *N. tabacum* plants (Vanhercke et al., 2014). The significance of this observation was accessed by a statistical analysis, which is further discussed below.

Combinatorial Gene Testing in Protoplasts Mimics Results Obtained With Leaf Transient Assay

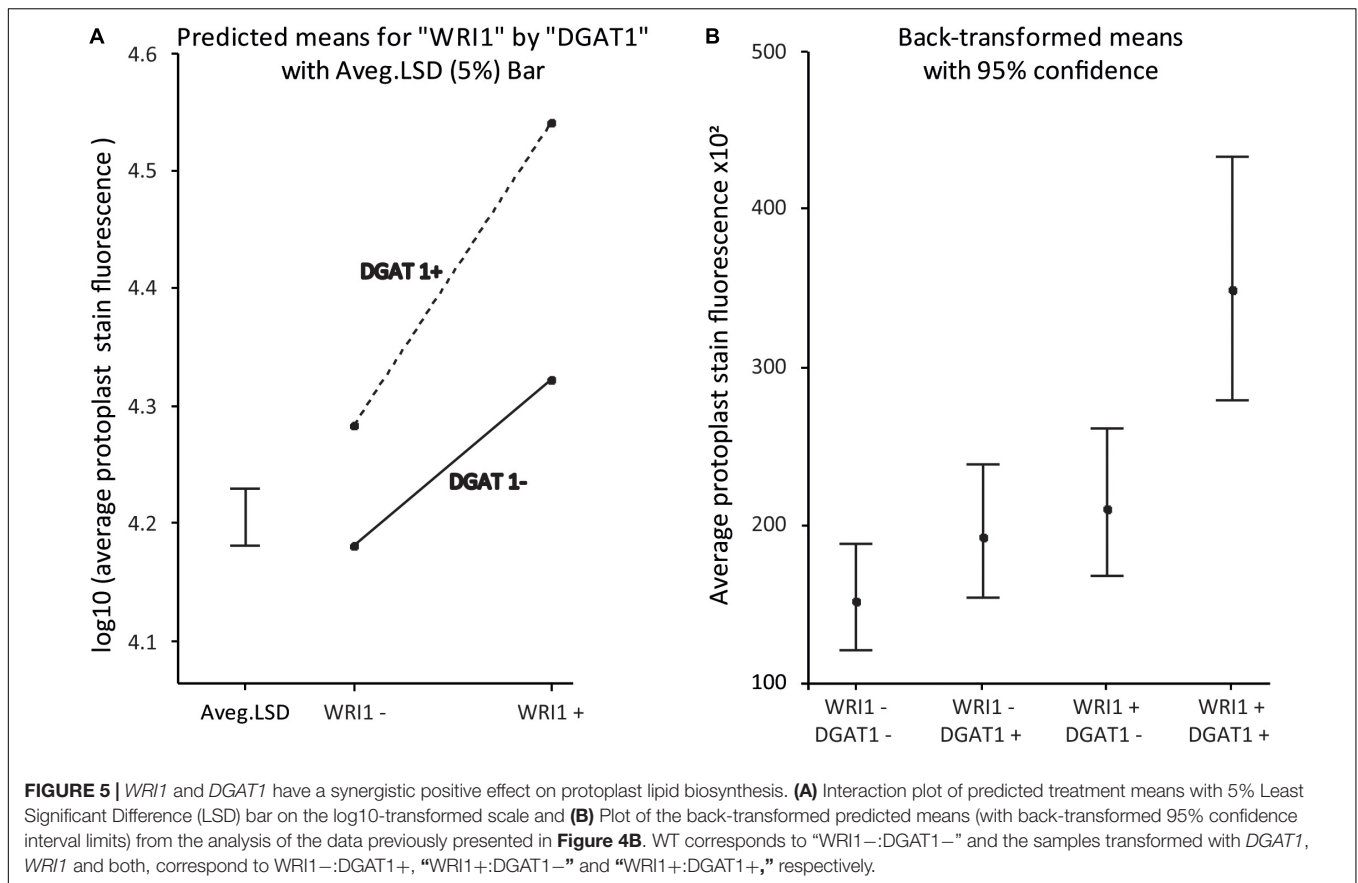
The statistical analysis of the data confirms the statistical significance of the synergistic effect, with a P -value for the interaction effect on the log₁₀-transformed scale of $p < 5e-6$. An interaction plot of the *WRI1* by *DGAT1* predicted means is given in Figure 5A. The significant P -value confirms that the lack of parallelism of the lines (and hence the presence of the synergistic effect) joining the pairs of means is genuine. Figure 5B plots the predicted means back-transformed to the scale of the data, with associated back-transformed 95% confidence interval limits for the means indicated by the error bars.

Overall our results confirm that protoplast systems are robust models that can be used to predict and inform plant phenotype and are compatible with combinatorial gene testing. This finding is particularly important for coupling transient protoplast expression with high throughput combinatorial gene screening.

ABI3 Induces Lipid Accumulation in Tobacco Leaf Protoplasts

Our new screening strategy constitutes a new high throughput tool to fast-track plant lipid engineering strategies and offers an alternative method to study plant lipid metabolism. We therefore wanted to use this newly developed strategy to investigate the relative effect of the major known seed specific developmental regulators. *ABI3*, *FUS3*, *LEC1*, and *LEC2* are master regulators that control the gene regulation networks governing most seed developmental mechanisms. *FUS3*, *LEC1*, and *LEC2* also have been reported to trigger oil accumulation through regulation of *WRI1*, which controls the expression of many genes essential for fatty acid synthesis. However, while many targets of *ABI3* were identified to function in seed oil storage, the direct effect of *ABI3* on lipid content in plants remained undocumented.

Tobacco leaf protoplasts were transiently transformed with *WRI1* and *DGAT1* only or with one of the master regulators, being *ABI3*, *FUS3*, *LEC1*, or *LEC2*. The effect on protoplast total neutral lipid content was then measured and compared as described above (Figure 6A). This first assay indicated that in addition to the combined effect of *WRI1* and *DGAT1*, only co-expression with *FUS3* and *ABI3* significantly (P -value < 0.05) further increased protoplast total neutral lipid content. This was particularly surprising due to the lack of a previously reported direct effect of *ABI3* overexpression on plant lipid accumulation. To date, the most efficient gene combination reported for lipid accumulation enhancement involved *WRI1* with *DGAT1* and *LEC2* or *SDPI* silencing (Vanhercke et al., 2017). Therefore, in a second similar analysis we investigated in more detail the individual effect of individual genes (*WRI1*, *DGAT1*, *LEC2*, and *ABI3*) and of several related combinations on total neutral lipid content in transiently transformed protoplasts (Figure 6B). This second assay confirmed that *ABI3* overexpression alone resulted in the highest accumulation of total neutral lipid in protoplasts. In addition, unlike *LEC2*, co-expression with *ABI3* further increased the lipid content of protoplasts transiently transformed with *WRI1* and *DGAT1*. This difference supports



the previous reports that *LEC2* regulates fatty acid biosynthesis mainly through the regulation of *WRI1* (Baud et al., 2007; Santos-Mendoza et al., 2008). Thus, the addition of *LEC2* might be ineffective due to the expression level of exogenous *WRI1* in the co-transformed protoplasts being already so high that triggering the expression of native *WRI1* would make a negligible effect on the overall *WRI1* expression levels in protoplasts. Overall, our results suggest that *ABI3* control over plant lipid metabolism and storage might involve mechanisms that act independently from the previously described *LEC2* and *WRI1* regulation networks. Our new screening approach also suggests that *ABI3* could be an important factor triggering oil accumulation in plants and suppose that *ABI3* should be of prime interest in future plant lipid engineering approaches.

DISCUSSION

Transient Protoplast Transformation as an Alternative Predictive Tool for Plant Lipid Engineering

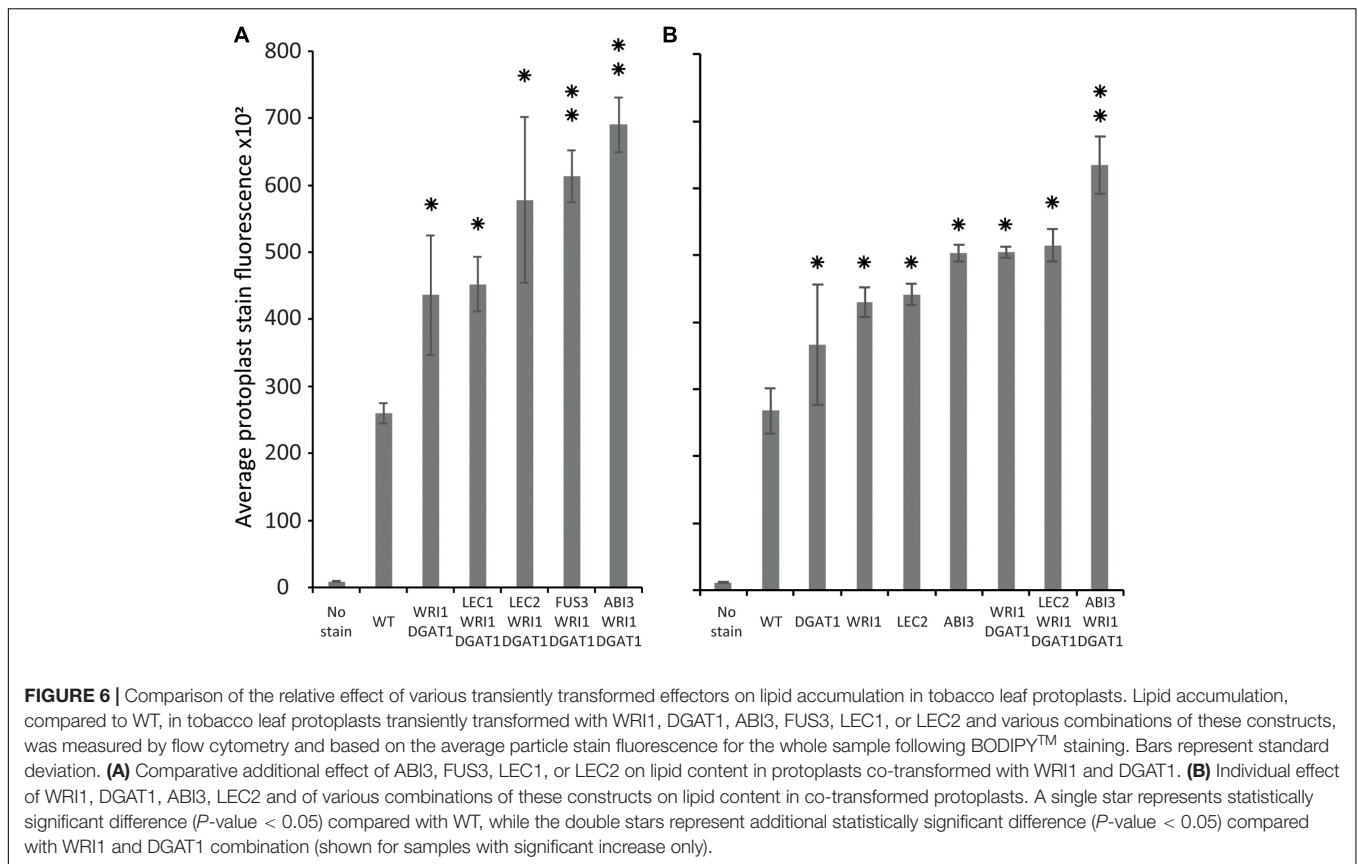
In this study, we demonstrated that protoplasts from 15-day-old tobacco leaves are a reliable model to study plant lipid engineering. This protoplast-based screening assay takes about three days to complete, as compared to the minimum of two weeks required for a traditional transient leaf transformation assays based on agroinfiltration and GC analysis. We confirmed

the direct correlation between protoplast lipid content and protoplast fluorescence intensity after lipid specific fluorescent staining. Furthermore, we show that protoplasts can be sorted based on lipid content and further used for downstream processing and lipid or molecular analyses. Our results demonstrate that transient gene expression in protoplasts produces lipid accumulation results similar to those obtained in stably transformed plants. We confirm that combinatorial gene testing is possible in protoplasts, with synergistic effect of the genes *WRI1* and *DGAT1* on increasing lipid accumulation in protoplasts, comparable to previously published studies conducted in transiently and stably transformed leaves (Vanhercke et al., 2013, 2014).

ABI3 Is a Major Regulator of Lipid Accumulation in Plant Cell

Using our newly developed workflow, we demonstrated that *ABI3* has a significant effect on protoplast total neutral lipid content. *ABI3* super-transformation significantly further increased the lipid content of protoplasts transiently transformed with *WRI1* and *DGAT1*, to a higher level than other known major effectors.

This result suggests that *ABI3* control over plant lipid metabolism and storage might at least partially involve mechanisms that are independent from the previously described *LEC2* and *WRI1* regulation networks. Our new approach also suggests that *ABI3* is a major regulator of oil accumulation in



plants and reveal ABI3 as a new target for future plant lipid engineering approaches.

A New High Throughput Platform for Gene Function and Plant Metabolic Engineering Study

The protoplast-based workflow presented in this study is very versatile and should be compatible with many crop species and for a wide range of future output traits. Indeed, protoplasts can nowadays be isolated from almost any plants and any tissue. A profusion of methods were developed during the 1970s and 1980s for many major crops and model plants, including tobacco (*N. tabacum*) (Takebe and Otsuki, 1969; Guerche et al., 1987a), cowpea (*Vigna unguiculata*) (Hibi et al., 1975), tomato (*Solanum lycopersicum*) (Motoyoshi and Oshima, 1979), turnip (*Brassica rapa*) (Howell and Hull, 1978), barley (*Hordeum vulgare*) (Loesch-Fries and Hall, 1980), cucumber (*Cucumis sativus*) (Maule et al., 1980) great millet (*Sorghum bicolor*), rice (*Oryza sativa*), wheat (*Triticum monococcum*) (Ou-Lee et al., 1986; Bart et al., 2006), canola (*B. napus*) (Guerche et al., 1987b), *A. thaliana* (Damm et al., 1989), and maize (*Zea mays*) (Sheen, 1990). Such approaches then fell into disuse probably due to the very high technical skills required to perform high quality protoplast isolation, transformation and culture. However, new methods were sporadically released for other crops of interest, such as carrot (*Daucus carota*) (Liu et al., 1994). Recently,

in the wake of a multitude of new high throughput cutting-edge technologies becoming easily accessible, protoplast-based systems have regained in their popularity and a profusion of improved methods have been released. Protoplast systems are the most suited for rapid screening of gene silencing (siRNA and miRNA) and genome editing (e.g., CRISPR) targets (Zhai et al., 2009; Cao et al., 2014; Maćkowska et al., 2014; You et al., 2014; Schapire and Lois, 2016). In addition, protoplast-based systems are now available for almost any plant of commercial interest, including lettuce (*Lactuca sativa*) (Sasamoto and Ashihara, 2014), grape (*Vitis vinifera*) (Wang et al., 2015) and bean (*Phaseolus vulgaris*) (Nanjareddy et al., 2016) and even the less expected, such as oil palm (*Elaeis guineensis*) (Masani et al., 2014), poplar (*Populus euphratica*) (Guo et al., 2015) and switchgrass (*Panicum virgatum*) (Burris et al., 2016).

In addition, our strategy should allow similar approaches for a broad variety of other metabolic traits since many fluorescent dyes, specific to particular metabolites are available. In the absence of a specific stain, genetic switches or biosensors can be developed for the specific detection of small molecules such as toluene (Behzadian et al., 2011), metabolites (Liu et al., 2017), and even specific proteins of interest (Abe et al., 2011; Wongso et al., 2017). For example, a new phosphatidic acid (PA) biosensor was recently developed to track PA cellular concentration and dynamics (Li et al., 2019). PA is the precursor of most phospholipid and triglyceride and represents around 1% of all lipid. PA acts as a key signaling lipid that predominantly

accumulates at the plasma membrane. This biosensor was based on the use of Förster resonance energy transfer (FRET) and was used to study the spatio-temporal complexity of PA accumulation in plant tissues and its contribution to mediate plant response to salt stress. Numerous other biosensors have been also developed based on FRET to study protein-protein, protein-DNA or DNA-DNA interactions (Lymperopoulos et al., 2010; Crawford et al., 2012). Alternatively, riboswitches have been engineered as nucleotide-based biosensors with great potential (Hallberg et al., 2017; Villa et al., 2018).

The new screening strategy presented in this study constitute the first step toward high throughput screening of complex genetic libraries in a plant system by using transient protoplast transformation and rapid cell sorting based on a fluorescent signal that is linked to a trait of interest. The workflow can be completed as a single experiment in a matter of days as opposed to several years that are needed by conventional means. Such novel synthetic biology inspired high throughput screens are paving the way for rapid identification of new unreported genes or gene combinations that significantly improve traits of interest in crops and thereby greatly improve the timelines associated with crop improvement (Zhang et al., 2019).

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**, excepted raw flow cytometry data.

AUTHOR CONTRIBUTIONS

BP, TV, and SS conceived the study. BP and CB performed the experimental work with assistance from HV for fluorescence

activated cell sorting. BP analyzed the data with assistance from AZ for statistical analysis. BP wrote the manuscript with contributions from all other authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2020.00288/full#supplementary-material>

FIGURE S1 | Repeat of thin layer chromatography plate presented in **Figure 2**. Thin layer chromatography plate separation of total fatty acids (TFA) extracted from leaves or from isolated protoplasts of WT or HO lines. STD stands for standards run in parallel of the samples to validate the location of the lipid.

TABLE S1 | Raw data for lipid quantifications presented in **Figures 2, 3**. Note: TAG/TFA content in tobacco leaf is presented in % of fresh weight leaf and in protoplasts in $\mu\text{g}/1\text{ M}$ cells.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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