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Chemical induction of the *Arabidopsis thaliana CBF1* gene in transgenic tomato fruit to study postharvest chilling injury

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ABSTRACT

Postharvest chilling injury (PCI) is a physiological disorder that contributes to the global loss and waste of fruit and vegetables. Ectopic expression of *C*-Repeat Binding Factor (CBF) transcription factors in tomato (*Solanum lycopersicum* L.) alleviates vegetative chilling injury, but their influence on PCI is unknown. We developed a dexamethasone (DEX)-inducible system with the aim of limiting high ectopic expression of *Arabidopsis thaliana CBF1* (*AtCBF1*) to fruit, and only during postharvest chilling. Our aim was (1) to optimize the DEX-inducible system for high *AtCBF1* expression in the organ and conditions of interest, i.e., fruit postharvest cold storage, and (2) to determine if *AtCBF1* could lessen PCI symptoms. Here, we describe the testing and validation of transformed lines from this system. In all tests, fruit were stored under PCI-inducing or non-PCI-inducing conditions. Across storage time, fruit immersion in 50 μM DEX for one hour prior to 2.5 °C storage or rewarming, induced *AtCBF1* expression 11- to 91-fold (mean: 33-fold, median: 29-fold), significantly higher than the 0.2- to 3.2-fold (mean: 1.5-fold, median: 1.3-fold) without DEX. Fruit color, surface pitting and decay incidence were used to indicate PCI. For genotype OE-8, PCI symptoms lessened during cold storage without DEX application compared to the wild-type. For line OE-2, DEX led to riper fruit at both 2.5 and 12.5 °C, while PCI-like symptoms developed under control temperatures (12.5 °C), after rewarming. In OE-6 fruit, DEX elicited a mild increase in the rate of ripening during the first week of cold storage compared to the wild-type. Our findings show that *AtCBF1* ectopic expression influenced fruit surface color or PCI symptom-development, and highlight that PCI is tightly interconnected with ripening. This is the first report of the use of a chemical-inducible system to ectopically express a gene to study a postharvest trait, and provides a basis for investigating the role of *CBF1* on PCI and ripening.

1. Introduction

Postharvest chilling injury (PCI) in tomato fruit negatively impacts fruit shelf-life and quality, and contributes to increasing postharvest loss and waste [3,9]. PCI has been extensively studied [42], but a comprehensive understanding of the molecular basis and mechanisms is still elusive.

The biological events that lead to the classic symptoms of PCI are amplified when previously cold-stored fruit are transferred to warmer conditions. These include visible phenotypes such as a failure of the fruit to ripen, development of surface pitting, and increased susceptibility to postharvest decay [12,14,42].

Unlike tomato fruit, the cold signal transduction pathway is better understood in the model species *Arabidopsis thaliana* [8,20]. A well-studied component of the cold response pathway in *Arabidopsis* is the *C*-Repeat Binding Factor (CBF) family of transcription factors [8,45,50–52]. The use of overexpression [18,19,60] and loss-of-function mutants [27,30,34,45,61] for *CBF1-3* in *Arabidopsis* has helped to elucidate their role in the development of cold tolerance in several species including tomato [11].

Ectopic expression of *AtCBF1* and its gene homologs has consistently led to increased freezing or chilling tolerance in a range of species besides *Arabidopsis* [25], including tomato [22,29], potato [38], and tobacco [58]. In tomato, transgenic plants expressing either *SlCBF1* (*35S::SlCBF1*) or *AtCBF3* (*35S::AtCBF3*) to high levels, induced the upregulation of genes that include dehydrin-like proteins, which protect against cold stress [60]. Nonetheless, the use of a constitutive promoter resulted in pleiotropic effects on plant growth and development [22] presumably

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due to CBF's interplay with the gibberellic acid (GA) pathway [1]. Previous work using a stress-inducible promoter (e.g. *RD29A*) provided mixed evidence on the effects of elevated levels of *CBF* expression in tomato plants. In some studies (*RD29A*::*AtCBF1*), these unintended effects were reduced [29,46], whereas in others (*RD29A*::*AtCBF3*), they were present, thus indicating leaky *CBF* induction [23]. However, to our knowledge, the chilling tolerance of the harvested product was not tested, and a potential role for *CBF* in PCI tolerance remains unresolved.

Limiting the ectopic *CBF* expression to harvested fruit during cold storage would help to isolate the effect of CBF1 on processes related to postharvest chilling injury. This would reduce the fitness cost associated with CBF1-induced physiological adaptation over the entire plant life-cycle [24,40,41], which may negatively affect postharvest processes. Fine control over gene expression could be accomplished through the use of a chemical-inducible gene expression system [33,36].

A synthetic, chimeric transcription factor GVG-construct was developed for the inducible expression of genes [7,64]. It consists of a DNA-binding domain cloned from the yeast transcription factor GAL4, the transactivating domain cloned from the herpes viral protein VP16, and the receptor domain of the rat glucocorticoid receptor called GR. This transcription factor is inactive in the cell, unless GR is bound by a synthetic glucocorticoid, in this case dexamethasone ('DEX'). When DEX is applied to the tissue and interacts with GR, the GVG transcription factor is able to enter the nucleus, and binds the Gal4 *cis*-element to drive expression of the target gene, in this case, *AtCBF1*.

There are no known reports where an inducible system was used to study physiological processes occurring postharvest, including PCI, in tomato. Therefore, it was first necessary to determine the conditions that would induce *AtCBF1* expression to levels that may influence fruit response after chilling. We conducted an exploratory study to establish the conditions for maximal expression of *AtCBF1* by DEX, in transgenic tomato fruit, during postharvest chilling. To maximize the specificity and control of the expression system, this construct should require two steps to elicit the transcription of *AtCBF1*: 1) cold exposure: this induces GVG transcription driven by the *RD29A* promoter, and 2) DEX application: this activates the GVG protein, allowing it to migrate to the nucleus to activate *AtCBF1* transcription. This study was done using one genotype, here named overexpression ('OE') line OE-8, under storage conditions that promoted mild, moderate and severe PCI. Then, we determined a DEX concentration and incubation time, to monitor changes in ripening and senescence, used as proxies for PCI, in three DEX-inducible genotypes i.e. OE-8, OE-2, and OE-6, and, wild type fruit. The results varied across genotypes but suggested that high ectopic *AtCBF1* expression could influence fruit phenotype postharvest.

2. Materials and methods

2.1. Seed germination

Solanum lycopersicum L. cv. Micro-Tom seeds were surface-sterilized in 3.7 % (v/v) sodium hypochlorite and two drops of Tween-20 (Sigma-Aldrich, Missouri, United States) for 30 min, and rinsed three times in 200 mL of sterile distilled water. Seeds were placed on half strength solidified agar, and half strength basal MS medium containing sucrose (15 g/L) and Phyto agar pH 5.6–5.8 (10 g/L) (bioWORLD plantmedia, Ohio, USA), followed by incubation at 26 °C for 7 days.

2.2. Construct development

The dexamethasone-inducible plasmid pTA7002 [7] was obtained from Dr. Nam-Hai Chua (The Rockefeller University, New York). The stress-inducible promoter *RD29A* (GenBank: CS191722.1) and the *AtCBF1* gene (GenBank: NM_118681.3) were separately cloned from *Arabidopsis thaliana* Col-0 genomic DNA (gDNA) (Table S1).

Fig. 1. Maturity stages of Micro-Tom tomato fruit. 'MG': mature green; 'B1': breaker 1; 'B2': breaker 2; 'T': turning; 'RR': red ripe; 'OR': overripe.

2.3. Plant transformation

Tomato cotyledons and hypocotyls were transformed with *Agrobacterium tumefaciens* EHA105 containing the plasmid *pTA7002-RD29A-AtCBF1* at the Plant Transformation Facility at UC Davis. Hygromycin B (20 mg/L) (Roche Diagnostics, Risch-Rotkreuz, Switzerland) was used as the selection agent, and successfully transformed plants were confirmed by PCR. Rooted plants (T_0 generation) were transferred to the greenhouse at UC Davis and grown at 25–30°C. Three independent homozygous transgenic lines (T_3 generation) were used in this study: 'OE-2', 'OE-6' and 'OE-8'. A wild-type or null-segregant ('NS') event that went through the tissue culture regeneration process was used as a genetic control.

2.4. Determination of transgene copy number

Genomic DNA was isolated from three biological replicates from homozygous, T_3 generation seedlings of OE-8, OE-6 and OE-8 [16]. Each replicate consisted of tissue from three seedlings. A genomic DNA dilutions series was prepared [44], with each dilution running in triplicate. PCR reactions were prepared as follows: 0.4 µL of water, 0.3 µL of 10 µM of each primer, 5 µL of iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) and 4 µL of diluted gDNA. Real-Time Quantitative PCR (CFX96 Touch™ Real-Time PCR Detection System, Bio-Rad, Hercules, CA, USA) was carried out under the following conditions: 95 °C for 3 min, and 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Reactions were run separately for each set of primers for *AtCBF1* and Prosystemin (*Prosys*), an endogenous control, and single-copy gene (Table S1) [17]. *AtCBF1* and *Prosys* primer efficiencies were 99 % and 97 %, respectively. The *AtCBF1* transgene copy number was estimated as the ratio of the copy number of *AtCBF1* to *Prosys* [44].

$$Ratio = \frac{1 + Efficiency_{AtCBF1}{}^{Ct\ AtCBF1}}{1 + Efficiency_{Prosys}{}^{Ct\ Prosys}}$$

2.5. Fruit harvest and phenotypic characterization

Unblemished fruit were harvested at breaker stage (Fig. 1), soaked in 0.25 % (v/v) sodium hypochlorite for three minutes and gently blotted dry with paper towels.

The width and height of 100 fruit per genotype were recorded. Fruit shape index (FSP) was the ratio of maximum fruit height to maximum fruit width. An FSP of 1, indicates round fruit; when FSP > 1 or FSP < 1, this indicated an elongated or squat fruit, respectively [10].

2.6. Postharvest assays: exploratory study of AtCBF1 ectopic expression on fruit postharvest chilling

Three storage temperatures were used: 2.5 °C (PCI-inducing temperature) or 12.5 °C (control, non-PCI-inducing temperature), followed by transfer to 20 °C for three days ('rewarming', 'RW') to induce PCI symptoms. A series of experiments were conducted to optimize the DEX system and assess fruit response to postharvest cold stress (Fig. 2).

2.6.1. AtCBF1 expression analysis of OE-8 fruit

Initial characterization of the DEX-inducible-*AtCBF1* expression system was performed on OE-8 in absence of the DEX inducer. Fruit at

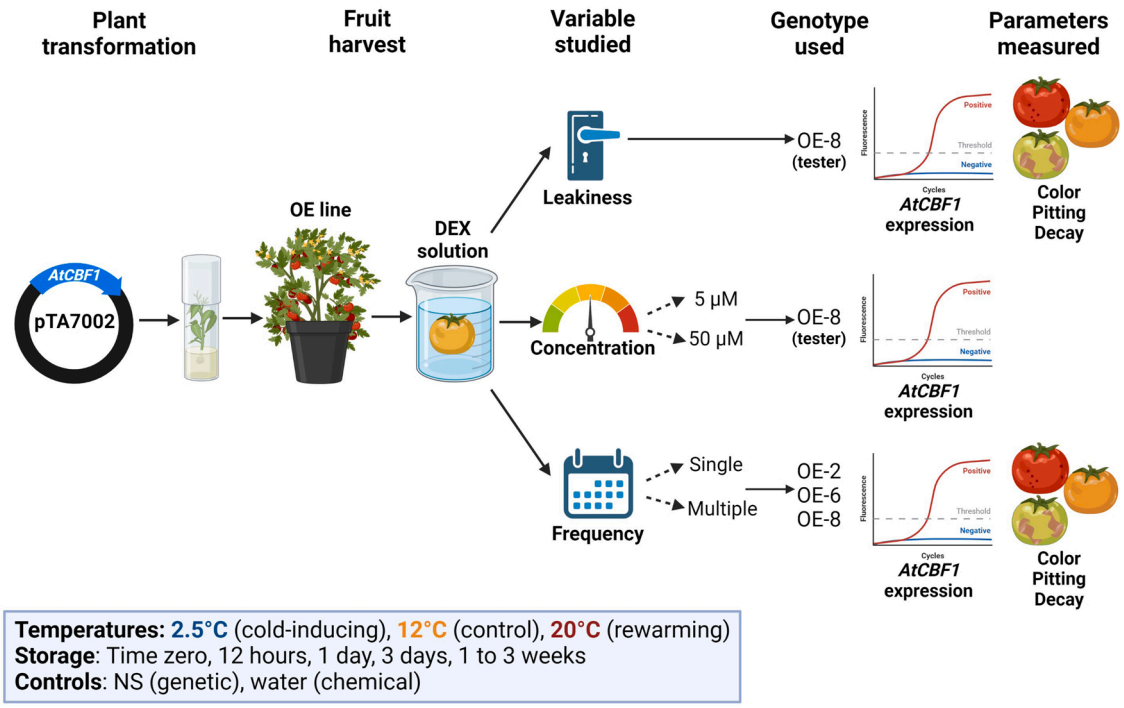

Fig. 2. Experiments for optimizing the DEX system and studying the fruit's response to cold stress postharvest. Line OE-8 was used as a tester to assess the DEX system's leakiness and optimal concentration. Further experiments included lines OE-2 and OE-6 in addition to OE-8.

breaker 2 stage were stored at 2.5 °C for one, two or three weeks, followed by RW. Objective color changes (Hue), decay percentage and Chilling Injury Index (CII) were monitored and contrasted with NS fruit. Expression of *AtCBF1* was measured by RT-qPCR.

2.6.2. *Influence of DEX concentration on AtCBF1 expression*

Breaker 2 fruit from line OE-8 were soaked at RT in: a) 5 µM DEX (SIGMA-ALDRICH, Missouri, USA) and the surfactant Silwet L-77 (LEHLE SEEDS, Texas, USA) at 0.01 % (v/v) for 1 h, dried and stored at 2.5 °C for up to 6 d, or b) 50 µM DEX (no surfactant) for 1 h, dried and stored at 2.5 °C for 24 h. Changes in *AtCBF1* transcript abundance were monitored by RT-qPCR.

2.6.3. *Impact of DEX application on different genotypes*

Fruit from lines OE-2, OE-8, and WT were harvested at breaker 1. OE-6 fruit were picked at breaker 2. All fruit were immersed in 50 µM DEX or water for 1 h at RT, dried and stored at 2.5 or 12.5 °C for one week, followed by RW. OE-8 fruit were incubated once. OE-2, OE-6 and WT fruit were re-incubated in DEX or water every three days until the end of the experiment. Repeated immersions were performed at the corresponding temperature (2.5, 12.5 or 20 °C). Hue, decay %, CII, and expression of *AtCBF1* by RT-qPCR were assayed.

2.7. *Chilling injury index (CII) and decay %*

Fruit were removed from the 2.5 or 12.5 °C rooms after 7, 14 or 21 d of storage, rewarmed for three days, and evaluated for CII based on the development of surface pitting. Pitting severity was assessed visually on each fruit using a five-point scale as follows: 0 = no pitting, 1 = < 25 %, 2 = 25–50 %, 3 = 50–75 %, 4 = > 75 % [2]. CII was calculated using the formula:

$$\%CII = \frac{\sum[(CII\ level)x(\#fruit\ \ at\ that\ CII\ level)]}{4xtotal\ \#fruit} x100$$

The percentage of fruit affected by decay after RW, was also recorded.

For the test on OE-8 fruit without DEX, and comparison against the NS, between 83 and 124 fruit were used. For tests involving DEX applications, 15 fruit were evaluated per treatment.

2.8. *Color determination*

The Hue angle was used as a descriptor of fruit ripening and measured by a Konica Minolta colorimeter (Chroma Meter CR-400, Konica Minolta Sensing Americas, Ramsey, NJ, USA) with a 2° observer and standard illuminant C setting in a three-dimensional color space using the L* a* b* scale. Higher Hue is associated with greener or less ripe fruit, whereas lower Hue values are indicator of redder or riper fruit. Readings were taken from the equatorial region of fruit pericarp. For testing OE-8 fruit in absence of the DEX inducer, and comparing it with the NS, 80 fruit were used. For tests involving DEX applications, 15 fruit were evaluated per treatment.

2.9. *Real-Time Quantitative PCR (RT-qPCR)*

Total RNA was isolated [56] from 100 mg of frozen fruit powder obtained from the pericarp region, according to Fig. 2, and treated with the DNase TURBO DNA free Kit (Life Technologies, Carlsbad, CA, USA). RNA integrity and purity were assessed by agarose gel electrophoresis, and ratios of A_{260}/A_{280} and A_{260}/A_{230}. One-microgram RNA was used for cDNA synthesis. RT-qPCR was performed as described in previous reports [3]. Primers were designed based on the cDNA sequences available on GenBank (Table S1). The reaction efficiency was calculated using the standard curve method, running a cDNA dilutions series [49]. Primer specificity was validated by melt-curve analysis [49]. The Pfaffl method was used for data normalization and relative quantification of transcript production [37]. Actin, *SlACT7*, was used as the housekeeping control for data normalization. Each treatment consisted of three biological and three technical replicates. A biological replicate corresponded to 3 or 6 fruit randomly harvested from 50 plants of the same genotype.

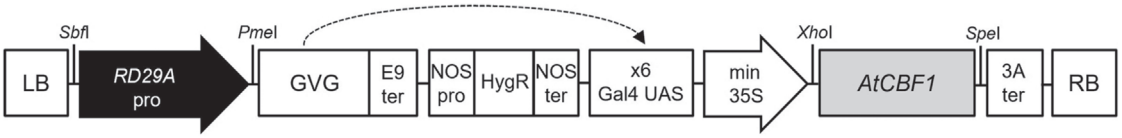

Fig. 3. The DEX-inducible *pTA7002-RD29A-AtCBF1* construct used for plant transformation. The construct consisted of the following from left to right. LB, Left T-DNA Border; *RD29A* pro, stress-inducible promoter from *A. thaliana*; GVG, chimeric transcription factor; E9 ter, transcription termination sequence; NOS pro, promoter from nopaline synthase; HygR, hygromycin resistance gene; NOS ter, terminator sequence; x6 Gal4 UAS, upstream activating sequence; min *35S*, minimal CaMV *35S* promoter; *AtCBF1*, *C*-Repeat Binding Factor 1 from *A. thaliana*; 3A ter, transcription termination sequence; and RB, right T-DNA border. Vertical lines represent the relative position of restriction sites used for cloning. The dashed arrow indicates that GVG binds to Gal4 UAS to induce *AtCBF1* transcription.

2.10. Statistical analysis

Experiments were organized as a complete randomized design. Statistical analysis was performed using SAS software Version 9.4 or Microsoft Excel. To detect significant differences between treatments, analysis of variance or unpaired *t*-test were conducted. Violin plot was graphed by the online platform https://www.bioinformatics.com.cn/en. For mean comparison, Tukey's test was used ($\alpha = 0.05$). The *z*-score test for two population proportions was used to analyze CII and decay between DEX and water treatments.

3. Results and discussion

To our knowledge there are no reports where 1) a dexamethasone system was employed to control the expression of a gene in fruit post-harvest, or 2) the overexpression of a member of the *CBF* gene family was used to study PCI in fruit. To date, overexpression of native tomato *CBF* genes in tomato has been scarcely explored [60], and most studies have ectopically expressed *CBF*s from *Arabidopsis* in tomato plants resulting in greater chilling tolerance in vegetative tissues [23,29,46,60]. Nonetheless, the impact of high *AtCBF* expression levels on tomato

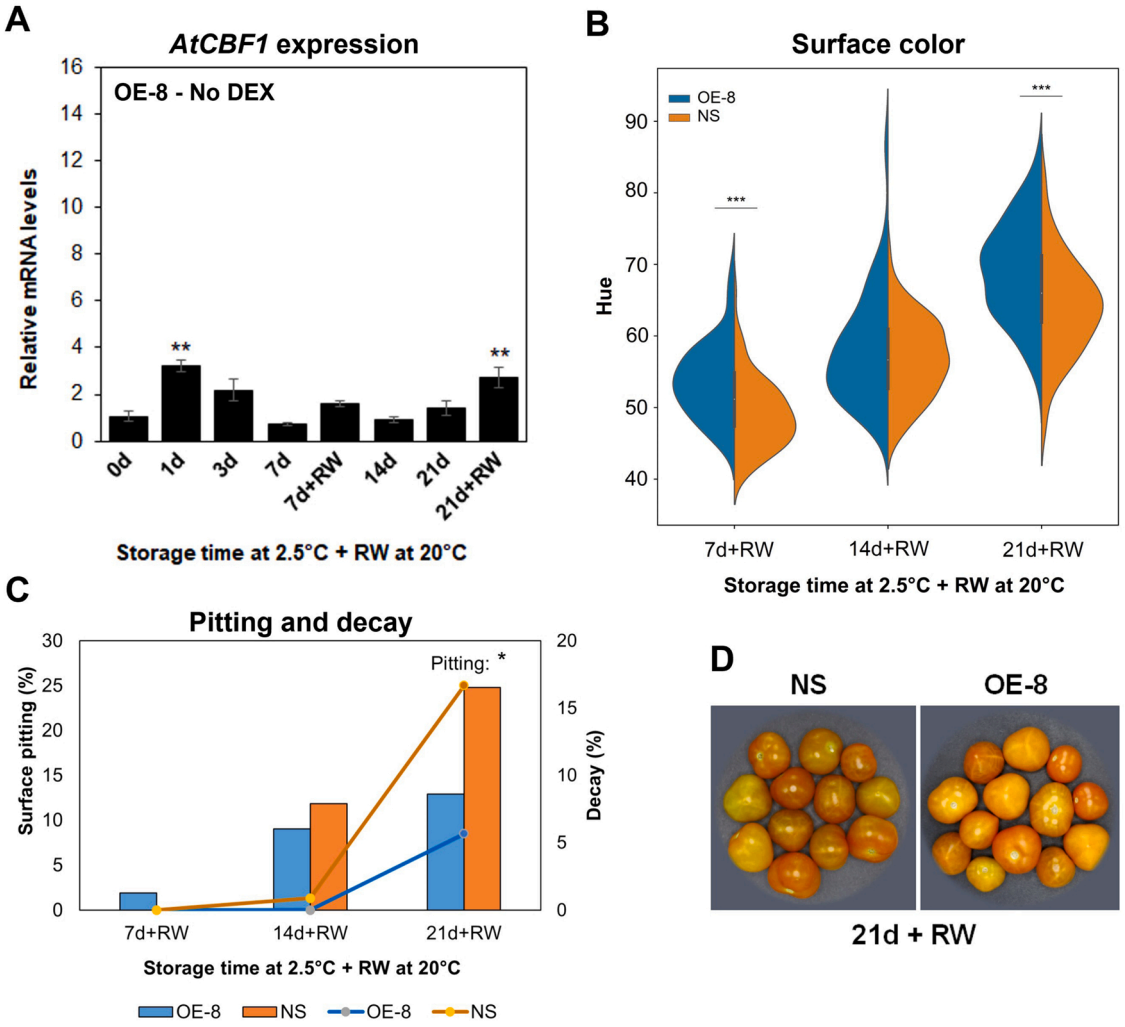

Fig. 4. *AtCBF1* expression in fruit during storage, fruit color, surface pitting and decay in absence of the DEX inducer. Fruit were stored at 2.5 °C, and then rewarmed, 'RW'. **(A)** Relative expression of *AtCBF1* in OE-8 fruit during storage compared to freshly harvested (0d) transgenic (OE-8) fruit, which was used as the control. Values are the mean ± standard error (SE) of 3 biological replicates, each consisting of 6 fruit. **($p < 0.01$), using an unpaired *t*-test. **(B)** Split violin plot of the color distribution of OE-8 and NS fruit during 2.5 °C storage, followed by RW. ***($p < 0.001$), using an unpaired *t*-test. **(C)** PCI incidence indicated by fruit surface pitting (bars) and decay (lines) after rewarming. *($p < 0.05$), using an unpaired *t*-test. **(D)** Visual changes in color in a representative sample of OE-8 and NS fruit.

fruit postharvest or PCI symptoms has been overlooked. Therefore, prior to characterizing the impact of *AtCBF1* ectopic expression on the development of PCI it was necessary to fully optimize the expression system, and to ensure that the construct was successfully transformed into the genome of select independent transformants. Several variables must be accounted for, including those related to transformation and regeneration, i.e., positional effects, and somaclonal variation, in addition to optimizing the activation of DEX-GVG-system and monitoring *AtCBF1* expression. Being able to connect the effect of *AtCBF1* expression to potential changes in tissue physiology may also be challenged by the high level of variation associated with studying postharvest processes [3,4].

3.1. Design of the chemically-induced expression system

The *RD29A* promoter was fused to GVG to limit the synthesis of this protein to stress (e.g. cold) conditions, since elevated levels of GVG due to constitutive overexpression have been linked to detrimental effects in plants [5,28]. The *RD29A* PCR product had *Sbf*I (5′ end) and *Pme*I (3′ end) restriction sites and was cloned into pTA7002 *Sbf*I and *Pme*I restriction sites by removing the *CaMV35S* promoter. The *AtCBF1* PCR product was amplified with *Xho*I (5′ end) and *Spe*I (3′ end) sites and cloned into the plasmid *Xho*I and *Spe*I sites downstream of *RD29A*. The resulting construct was named *pTA7002-RD29A-AtCBF1* (Fig. 3).

To confirm the presence of the *pTA7002-RD29A-AtCBF1* construct in the putative transgenic lines, a fragment spanning the NOSter and the *AtCBF1* gene was amplified (Fig. 3).

The nucleotide and amino acid sequences of *AtCBF1* (GenBank: NM_118681.3) and the native *SlCBF1* (GenBank: NM_001247194.2) gene share a 57 % and 58 % identity, respectively (Fig. S1) , according to the Multiple Sequence Alignment by CLUSTALW tool [53]. In order to avoid the co-amplification of the native *SlCBF1* while determining *AtCBF1* expression level and transgene copy number, primers were designed based on non-conserved regions presenting polymorphisms between both sequences (Fig. S1). In addition, *AtCBF1* expression was tested in non-transgenic (NS) fruit via RT-qPCR, and no transcripts were detected.

3.2. OE-8 fruit responded favorably to PCI-inducing conditions in absence of the DEX inducer

The DEX-optimization and initial characterization of the *AtCBF1* transgenic lines was carried out using an independent transformant designated OE-8. We first determined if there was more than one copy of the plasmid transformed into OE-8. Transgenic events with a single transgene copy are desired since segregation is likely to occur in a Mendelian fashion. Moreover, the chances of transgene silencing or simultaneous deletion of the inserted fragment are lower than in events with multiple copies [13,55]. Real-Time Quantitative PCR analysis of OE-8 genomic DNA suggested that there was only one copy of the transgene (Table S2).

Fruit size and shape, expressed as the 'Fruit shape index' differed between non-transgenic and transgenic samples (Table S3). Non-transgenic fruit, herein called 'NS', obtained from a null-segregant event, were used as a genetic control to perform comparisons against OE-8.

It was important to see the physiological changes in OE-8 and NS fruit after cold-storage where they were not immersed in DEX. Ripening and PCI symptoms were evaluated based on fruit color, surface pitting, and decay. Surface color is a valid descriptor for quality, as pigments, such as carotenoids and chlorophylls contribute either directly or indirectly to visual appeal, taste and flavor [6]. For OE-8, putative *AtCBF1* expression was also determined at seven timepoints over a three week period.

In the absence of the chemical inducer, *AtCBF1* expression during refrigeration was the same as that in OE-8 fruit before cold-storage at five of the seven time points assayed, except a) after one day of cold-storage, when it increased 3-fold, and b) when fruit were rewarmed after three weeks in the cold (2.7-fold) in OE-8 (Fig. 4A).

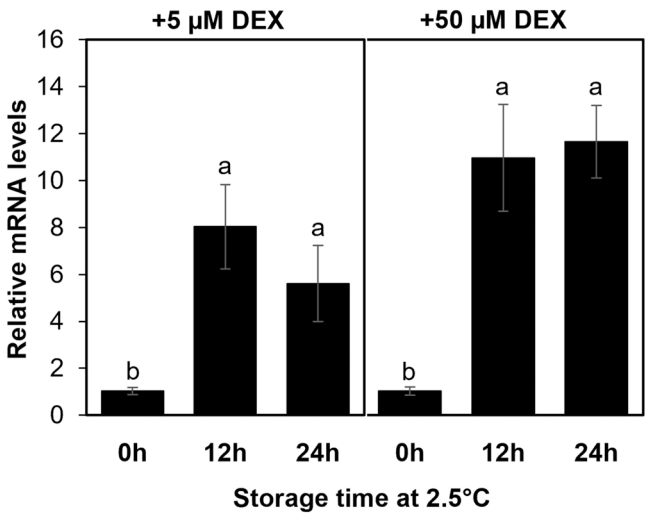

Fig. 5. Relative *AtCBF1* expression in OE-8 fruit with different DEX concentrations, during cold storage. Freshly harvested fruit were immersed in 5 or 50 µM DEX for 1 h. Untreated, transgenic fruit (0 h) were used as the calibrator. Values are the mean ± SE of 3 biological replicates, each consisting of the pericarp region of three (5 µM) or six (50 µM) fruit. Different letters indicate significant difference ($p < 0.05$) between time points within each treatment by Tukey's test. Additional expression data can be found in 'Supplementary material'.

OE-8 showed signs of chilling injury, i.e., reduced ripening and brown areas on the fruit surface after two weeks of cold storage (Fig. 4B). However, after an additional week of cold storage, followed by rewarming, OE-8 showed less PCI symptoms and had a better overall appearance compared to the NS fruit in terms of surface pitting ($p = 0.019$), and decay incidence ($p = 0.13$) (Fig. 4C, D).

3.3. DEX induced AtCBF1 transcript accumulation in a dose-dependent manner in OE-8 fruit

The response of the transgenic fruit to two different DEX concentrations, 5 and 50 µM, was tested in OE-8, and *AtCBF1* expression was monitored during short i.e., 2–12 h, 1 day, or longer-term, i.e., 7 days, cold storage. These DEX concentrations are within a range tested previously in species such as rice [35], *Arabidopsis* [32] and *Medicago truncatula* [31]. Fruit were immersed in DEX for 1 h, consistent with the range of incubation times used in studies where exogenous chemicals were applied to assess either ripening [47,48] or PCI [2,26,63] in tomato fruit.

Treating fruit with 5 µM DEX resulted in a 5- to 8-fold upregulation of *AtCBF1* when assayed at the first timepoint, i.e., 12 h (Fig. 5), a change that did not occur in the absence of DEX (Fig. 4A). *AtCBF1* transcripts remained at this level for 7 days in the cold (Fig. S2). A treatment of 50 µM DEX was then chosen to determine if a higher dose could modify *AtCBF1* expression [57], since the pericarp cuticle could interfere with DEX penetration and diffusion to the inner fruit tissues. At this concentration, the transgene was induced to slightly higher levels (11-fold) compared to the 5 µM treatment (8-fold) after 12 h (Fig. 5). Consistent with other reports, the concentration range used here positively correlated with transgene expression [57].

In addition to a dosage effect, a fast induction system would enable more precise tuning of target protein accumulation [43]. In this study, we showed that the DEX-inducible expression system responded within two hours of chemical treatment and cold storage (Fig. S2).

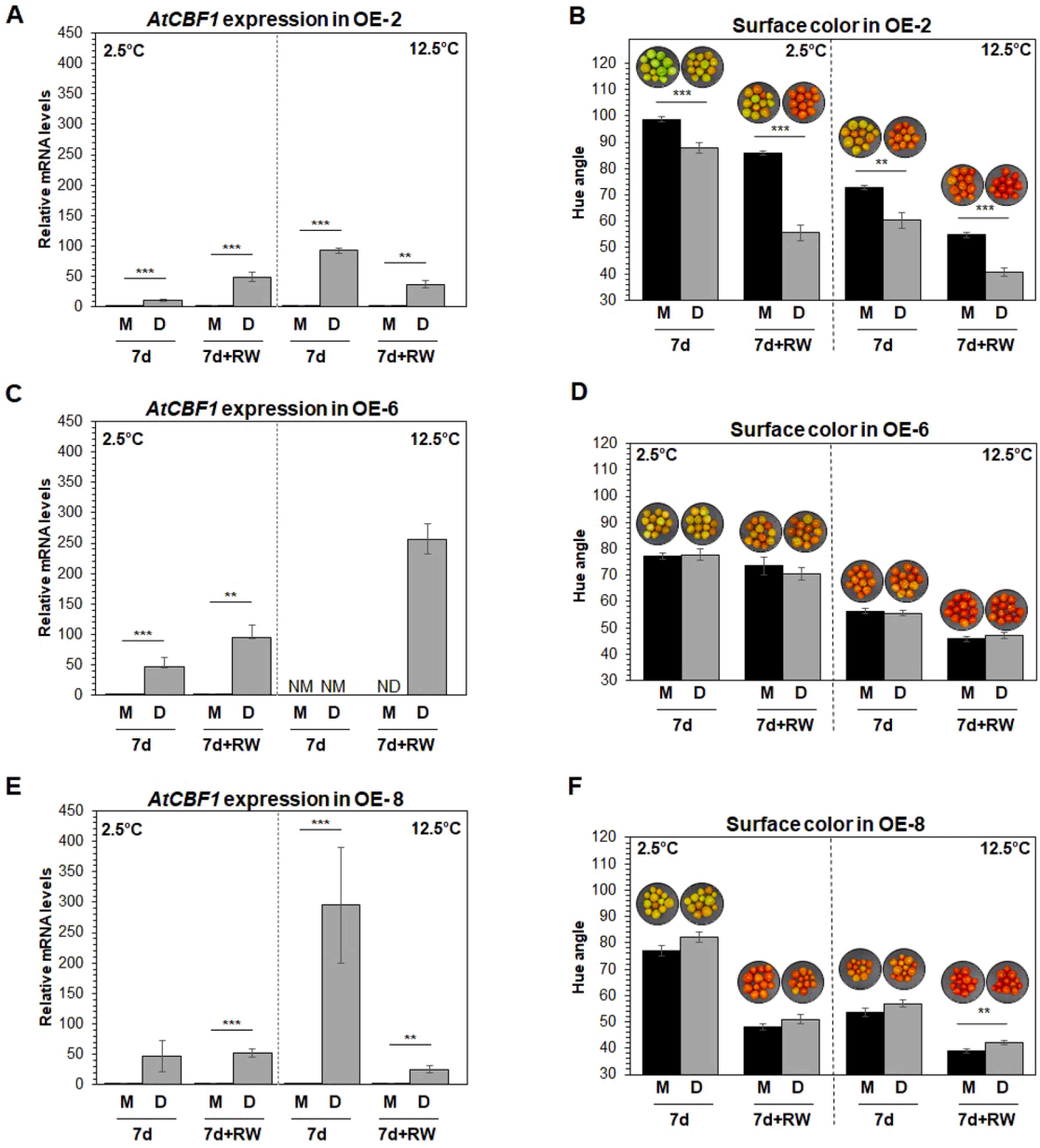

Fig. 6. Surface color and *AtCBF1* expression in transgenic fruit treated with water or DEX during storage. Freshly harvested fruit were treated with water (mock, 'M') or 50 μM DEX ('D') for 1 h at RT, and stored at 2.5 or 12.5 °C for 7 days followed by rewarming 'RW' for 3 days**.** DEX was applied every three days until the end of the experiment in OE-2 and OE-6, but performed once in OE-8 fruit. Columns with asterisks are significantly different ($p < 0.05$) by unpaired *t*-test. **(A)**, **(C)**, **(E)** Relative *AtCBF1* expression. Freshly-harvested transgenic, untreated fruit were used as the calibrator. Values are the mean ± SE of 3 biological replicates, each consisting of the pericarp region of 6 fruit. Transcript levels not detected and not measured are denoted as 'ND' and 'NM', respectively. **(B)**, **(D)**, **(F)** Hue angle. Each column is the mean ± SE of 15 fruit.

3.4. Responses of independent transgenic lines to applications of the DEX inducer

To assess the effect of DEX on fruit biology post-harvest, freshly harvested NS fruit were exposed to single or multiple DEX applications and stored at control or PCI-inducing conditions. Overall, DEX had no effect on ripening (Fig. S3), decay incidence or CII scores (Fig. S5) in non-transgenic fruit, as these data were similar to the water-control. This confirms that DEX did not induce toxicity or alterations in ripening progression. Lack of toxicity is a basic requirement of a chemically-regulated gene expression system [36,43]. To explore potential variability across independent transformation events in response to DEX, two independent, homozygous transformants in addition to OE-8, named OE-2 andOE-6, were studied. Estimation of transgene copy number by RT-qPCR revealed that OE-2 and OE-6 had two T-DNA copies, unlike OE-8, which was a single transgene-copy event (Table S2).

Fruit of each genotype were immersed in water ('mock') or in 50 μM DEX once (OE-8), or every three days (OE-2 and OE-6). Color, PCI development and *AtCBF1* expression were monitored during storage at 2.5 or 12.5 °C for one week, followed by rewarming for three days.

Water treatment did not induce *AtCBF1* expression to significant levels in the transgenic genotypes. Transcript level in control-stored fruit was 0.2–1.4-fold, and in cold treatment values were similar, i.e., 0.2–2.1-fold expression (Fig. 6A, C, E). Low basal levels of target gene expression is one of the main requirements of an inducible expression system, to be useful for investigating biological processes [57], such as, postharvest chilling injury. Here, we show a chemical-inducible system with minimal transgene background expression in the absence of DEX.

Overall, there were no differences in decay incidence or surface pitting that could be associated with a higher application frequency of DEX in either of the transgenic genotypes (Fig. S5). This could be attributed to the relatively short cold exposure used in these tests i.e., 1 week. However, changes in fruit color are likely related with the maturity stage at harvest that was used for the experiments in OE-6, or a genotype-specific effect in OE-2 (Fig. S4).

AtCBF1 was upregulated in all transgenic genotypes, in response to DEX, regardless of temperature (Fig. 6A, C, E). Transgene expression was higher in fruit treated with DEX and stored in the cold, when compared to untreated-freshly-harvested fruit at 0 d (Table S4). Transgene expression was also higher at each timepoint during cold-storage when fruit treated with DEX was compared to mock-treated transgenic fruit (Table S5).

Remarkably, storage at 12.5 °C alone or followed by rewarming, was associated with the highest levels of *AtCBF1* expression in all genotypes, i.e., 91-fold in OE-2, 245-fold in OE-6, and 306-fold in OE-8 (Fig. 6A, C, E). Previous research indicate that in WT, non-transformed tomato, higher levels of *SlCBF1* expression occurred in fruit stored at 12.5 °C followed by RW, in contrast to storage at 2.5 °C plus RW, which suggests that *CBF* may have a role in fruit ripening [3]. This has not been explored to date, but some data support this observation [15,59,62].

The ripening phenotype of the transgenic lines was variable and did not always correlate with *AtCBF1* expression. In OE-6 and OE-8 fruit, DEX did not cause changes in fruit color (Fig. 6D, F), even when *AtCBF1* expression was abnormally high, i.e., 245- to 300-fold that of the gene in water-treated fruit (Fig. 6C, E). In contrast, in DEX-treated OE-2, the rate of ripening was accelerated when compared to water-treated fruit, within one week of storage, at both control and chilling temperatures (Fig. 6B). This ripening acceleration correlated with the high levels of *AtCBF1* transcript, 91-fold, observed after the DEX application (Fig. 6A). Due to variable fruit availability, OE-6 fruit was harvested at breaker 2 instead of breaker 1 as with the other genotypes, and this likely influenced postharvest ripening in response to DEX (Fig. S4). Previous research has shown that tomato *SlCBF1* expression varies as a function of fruit maturity stage and cold storage time [62]. Therefore, an interaction between the pathways regulated by the tomato's endogenous *CBF* genes and *AtCBF1* cannot be ruled out.

The dissociation between *AtCBF1* expression and fruit color in OE-6 and OE-8 could be explained by, a) transcript abundance that saturated the system, such that the response was not proportional to the level of *AtCBF1*, potentially associated to the presence of two copies of the transgene, or b) triggered posttranscriptional regulatory mechanisms to reduce *AtCBF1* protein levels [54]. Further experiments involving longer postharvest storage times e.g. two to three weeks of cold storage, will be required to elucidate the relationship between *CBF1* expression and ripening during cold storage. Moreover, next research steps should include measuring *AtCBF1* protein abundance in OE lines.

Phenotype variation among the transgenic lines could be associated with the location where the transgene integrated into the genome i.e., positional and insertion effects, which has been reported by other authors [21,39].

4. Conclusions

PCI is a complex disorder that affects numerous species of economic interest from tropical and sub-tropical regions, including tomato fruit. Our aim was to develop a DEX-chemical inducible system that would allow us to elicit high levels of ectopic *AtCBF1* mRNA in tomato fruit after harvest, in a highly controllable manner. Limiting *AtCBF1* expression to the fruit and amplifying expression during cold storage would allow us the test the hypothesis that CBF1 could improve fruit tolerance to PCI. Because there are a multitude of factors that affect physiological processes in postharvest fruit, such a system would require extensive optimization. We successfully identified the conditions, i.e. DEX concentration and application frequency, that achieved remarkably high, i.e., 11–91-fold *AtCBF1* expression, and this was sustained over one week storage in the cold. Multiple factors, i.e., positional effects, somaclonal variation, differences in transgene copy number, and fruit development at harvest converged to weaken the relationship between DEX-induced *AtCBF1* expression, and ripening among the independent transgenic events. However, this work is the only report to date on postharvest elicitation of ectopic gene expression.

CRediT authorship contribution statement

Karin Albornoz: Methodology, Investigation, Formal analysis, Validation, Writing – original draft. **Jiaqi Zhou**: Methodology, Investigation. **Diane M Beckles:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cpb.2023.100275.

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