דו"ח מסכם לתוכנית 203-0870-13

סירוגיות בהדרים: לימוד תהליכים המשתנים בין שנת שפע לשנת שפל

Alternate bearing in citrus

מוגש לקרן המדען הראשי במשרד החקלאות ופתוח הכפר

ע"י

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תקציר

הצגת הבעיה: סירוגיות ביבולים, המתבטאת ביבול נמוך בשנה אחת ויבול רב בשנה העוקבת, מהווה בעיה בעצי פרי, ובכללם הדרים. המנגנון (ים) הקובעים סירוגיות אינם ברורים. ניתן להניח כי עומס היבול גורם לשינוי במצב הפקע וברמת מוכנותו לקבל את סיגנל הפריחה בתקופת האינדוקציה לפריחה (טמפרטורה נמוכה). מטרות המחקר: 1. ביצוע אנליזה גנומית משווה של פקעים מעץ בשנת שפע ממנו מוסר הפרי לעומת עץ ביקורת. 2.אימות תוצאות של האנליזות הגנומית ברמה המולקולארית והביוכימית. 3. בחינת האפשרות כי הגן ההומולוגי ל-*SPL*, גן המבקר פריחה עונתית דרך בקרת גנים לפריחה, משחק תפקיד בבקרת הסירוגיות. שיטות המחקר: ביטוי גנים בוצע ע"י Real time PCR, האנליזה הגנומית בוצעה ע"י RNA deep sequencing, ואימות התוצאות ע"י Real Time PCR, Western Analysis, ואנליזה כימית של הורמונים. כמו כן, בוצעה התמרה לארבידופסיס ולהדרים.

תוצאות: תוצאות האנליזה הגנומית הראו כי פקעי עצים מהם הוסר הפרי הופכים להיות דומים לפקעי עצי שפל כבר שבוע לאחר הסרת הפרי. אובחנה עלייה במערכת הפוטוסינטתית בפקעי שנת שפל ובפקעי עצים מוסרי פרי לעומת פקעי שפע. אופיינה עליה חדה בביטוי גנים הפעילים בטרנספורט פולארי של אוקסין התלוי בסידן בפקע לאחר הסרת הפרי. אנליזה של

רמת ההורמון אוקסין הראתה ירידה ברמתו בפקעי שנת שפל ובפקעי עצים מוסרי פרי לעומת פקעי שפע. אובחנה עלייה בגנים לביוסינתזה של ההורמון ABA, אשר לוותה בירידה ברמת ההורמון בפקע לאחר הסרת הפרי. דגם הביטוי של גן זה בהדרים ובתפוח מציע כי הוא עשוי לשחק תפקיד בבקרת הסירוגיות. הגן מהדרים הביא להקדמת פריחה בארבידופסיס, אולם, עד כה, ביטויו ביתר בהדרים, לא הביא לפנוטיפ הצפוי. במסגרת המחקר נבחנה האפשרות כי האזור בו מצוי הגן *SPL* בגנום ההדרים עובר שיעתוק מורכב שעשוי לשחק תפקיד בבקרת הגן.

<u>מסקנות והמלצות</u>: לסיכום, תוצאות המחקר מרמזות על חשיבות ההורמונים אוקסין ו-ABA בקביעת רמת מוכנות הפקע לקבל את סיגנל הפריחה כתלות בעומס הפרי. על בסיס התוצאות, מבוצעת במעבדה עבודה פיזיולוגית שמטרתה לבחון את ההיפותזות שעלו במסגרת התכנית.

הצהרת החוקר

הממצאים בדו"ח זה הנם תוצאות ניסויים, הניסויים אינם מהווים המלצות לחקלאים

רשימת פרסומים שנבעו מהמחקר

במאמר השני, רק החלק העוסק ב-*SPL* הנו חלק ממחקר זה, בעוד ששאר החלקים הנם חלק מתכנית קודמת שמומנה.

1. Shalom, L, Samuels S, Zur N, Shlizerman L, Faigenboim A, Blumwald E, Sadka A (2014) Fruit load induces changes in global gene expression and in ABA and IAA homeostasis in citrus buds. *J. Exp. Bot*. Published On Line. doi:10.1093/jxb/eru148
2. Shalom, L., Samuels, S., Zur, N., Shlizerman, L., Zemach, H., Ofir, R., Blumwald, E. and Sadka, A. (2012). Alternate bearing in citrus: changes in the expression of flowering control genes and in global gene expression in ON- versus OFF-crop trees. *PLOS ONE,* 7(10): e46930. doi:10.1371/journal.pone.0046930

חתימת החוקר:_____ תאריך: <u>06/06/14</u>

תוכן עניינים

מבוא

סירוגיות הנה מהבעיות החמורות בענפי המטע השונים, ובכלל זה בפרדס (Monselise and Goldschmidt 1982). בזני הדרים שונים היא מופיעה בעוצמות שונות. בזנים מורקוט ווילקינג משרעת היבול בין שנת שפל לשנת שפע הנה גדולה מאוד, ודווחו מקרים בהם לאחר שנת שפל עם פירות בודדים לעץ, בשנת השפע עצים התמוטטו מעודף יבול כאשר לא בוצע דילול. בזנים אחרים, כמו שמוטי, הסירוגיות מופיעה בעוצמה נמוכה, וההבדל בין שנת השפע לשנת השפל הנו נמוך יחסית. בזנים אחרים, כמו קומקוואט, מופיעים יבולים כבדים שנה אחר שנה, ואם יש סירוגיות היא כמעט אינה מורגשת. בצורתה הקלאסית, הסירוגיות מופיעה בשלב הפריחה, כאשר בשנת השפל יש הפחתה באינדוקציה לפריחה, וכפועל יוצר מכך מספר הפרחים נמוך יותר וכך גם היבול. בחלקות רבות של אור הסירוגיות אכן מופיעה בצורתה הקלאסית, אבל ישנן חלקות בהן יש סירוגיות לא סדירה, למשל שנתיים שפע אחרי שנת שפל, וההיפך. מעבר לבעיית מספר הפירות, בשנת השפל חלק נכבד מהפרי בהדרים הנו גדול מדי, ונוטה להיות בעל קליפה עבה, ולכן נדחה לייצוא בבית האריזה. בשנת השפע הפרי קטן מדי ובעל איכות שיווקית נמוכה. הגישות ההורטיקולטוריות להפחתת תופעת הסירוגיות מתחלקות לכאלו שמטרתן לעודד פריחה בשנת השפל, לכאלו המבוססות על דילול חנטים בשנת השפע, וכן לגישות המבוססות על עיצוב העץ בשיטות שונות. שימוש במעכבי ביוסינטזה של ג'יברלינים, בעיקר פקלובוטרזול (קולטאר), ע"מ לעודד פריחה בשנת השפל נוסה בהדרים, אבל, בניגוד לאבוקדו, הוא לא מיושם במטע בעיקר בגלל אפקט שאריתי ארוך טווח שהביא לתוצאות לא רצויות כמו הקטנת גודל פרי (Greenberg et al., 1993). בנוסף, החומר עודד היווצרות של תפרחות טהורות (חסרות עלים) אשר החנטים הנוצרים מהם נוטים לנשור. גם שימוש בג'יברלין בשנת השפע ע"מ להקטין מספר פרחים אינו מקובל בהדרים, מאחר ומקובל דילול חנטים בשלב יותר מאוחר ע"י תרכובות אוקסיניות, אשר יעילותן הוכחה הן כמדללות והן כמגדילות פרי (Erner et al., 1993; Guardiola and Garcia-Luis 2000). ניסויים שונים הצביעו על כך שחיגור סתווי יעיל גם הוא לעידוד פריחה בשנת השפל (Goren et al., 2003). מספר חקלאים נוקטים בגישה זו, אולם ככלל, האמצעי הזה אינו נפוץ. שימוש באמצעים מעצבי נוף: גיזום ענפי קיץ, כיפוף ענפים או קיטומם פורסמו כאמצעים להקטנת סירוגיות (Bowman, 1999), אולם גם הם לא מצויים בשימוש נרחב. לסיכום, האמצעי היעיל ביותר הנו דילול חנטים באוקסינים מיד לאחר סיום הנשירה הטבעית.

הגורמים האחראים על הופעת הסירוגיות אינם ברורים. בספרות מופיעות שתי תיאוריות עיקריות, התיאוריה התזונתית, הטוענת כי לאחר שנת שפע רמת הפחמימות בעץ יורדת, ונוצר סיגנל המשפיע באופן שלילי על האינדוקציה לפריחה (Goldschmidt et al. 1985; Li et al. 2003). התיאוריה השנייה טוענת כי בתקופת השפע נוצר מעכב (כי הנראה חומר צמיחה) המשפיע שלילית על האינדוקציה בשנה העוקבת (Talon et al. 1997). עדויות לתקפות תיאוריה זו או אחרת מצויות בספרות, אולם מדובר לרוב בעדויות נסיבתיות אשר אינן יורדות לעומק המנגנונים. סביר להניח כי האינדוקציה לפריחה מושפעת מגורמים שונים, כולל גורמים תזונתיים, וכן מגורמי צמיחה שונים.

תכנית זו הנה תכנית המשך לתכנית קודמת בה ניסינו לזהות את הגורמים המבקרים סירוגיות בעצי הדר, דרך אפיון שינויים בביטוי גנים מבקרי פריחה, וכן שינויים גלובליים בביטוי גנים בין פקעים וענפים + עלים של עצי שנת שפע לעצי שנת שפל, וזאת תוך שימוש ב-microarray של Affymetrix. האנליזה הביאה למספר תובנות שהיוו בסיס לתכנית ההמשך הנוכחית:

1. שינויים נרחבים בביטוי גנים בין פקעי שנת שפע לפקעי שנת שפל מופיעים כבר בחודש מאי, לאחר הפריחה והחנטה. לעומת זאת, ההבדלים בביטוי גנים בתקופה יותר מאוחרת, חודש ספטמבר, היו מועטים יחסית.
2. זוהה גן שהראה ביטוי גבוה באברי עצי שפל לעומת עצי שפע בכל נקודות הזמן שנבחנו. גן זה הראה הומולוגיה לגן *SPL5-like* מארבידופסיס, גן-על המבקר פריחה דרך בקרת ביטויים של גנים אחרים, כמו *FT* ו-*Leafy*, ומבוקר בעצמו ע"י miR156 (Corbesier and Coupland 2005).
3. אופיינה עליה בביטוי גנים פוטוסינטתיים בפקעי שנת שפל לעומת פקעי שפל. כמו כן, זיהנו בפקעי עצי שפע עלייה במטבוליזם של טרהלוז, סוכר המשחק תפקיד בבקרת פריחה בארבידופסיס ובצמחים אחרים.

לאור הממצאים הנ"ל, במסגרת מחקר ההמשך נבחנו שתי היפותזות עבודה. האחת, שינויים חשובים בביטוי גנים המופיעים בפקעים בחודש מאי, לאחר הפריחה והחנטה, והקשורים למצב הסירוגיות של העץ, יופיעו בשלב מאוחר יותר, עם הסרת פרי והכנסת עצים בשנת שפע לשנת שפע עוקבת. השנייה, לגן *SPL5-like* חשיבות בבקרת הפריחה החוזרת, וה-*miR156* עשוי לשחק תפקיד בעיכוב הפריחה החוזרת לאחר שנת שפע.

מטרות המחקר

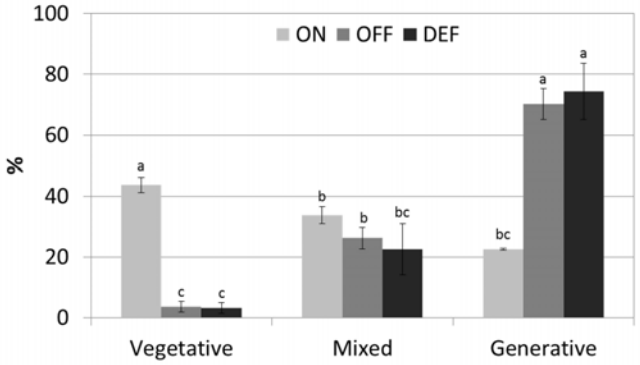
1. ביצוע אנליזה גנומית משווה של פקעים מעץ בשנת שפע ממנו נקטף הפרי לעומת עץ ביקורת. השוואת אנליזה זו לתוצאות האנליזה הקודמת תעזור להתמקד באותם תהליכים שעובר העץ במעבר משנת שפע לשנת שפל. האנליזה תלווה בבחינה מיקרוסקופית ומדידות של הפקעים לאחר הסרת הפרי ע"מ לאבחן שינוי בגודל.
2. אימות תוצאות של האנליזות הגנומית ברמה המולקולארית והביוכימית.
3. בחינת האפשרות כי הגן ההומולוגי ל-*SPL3* משחק תפקיד בבקרת הסירוגיות

פירוט עיקרי הניסויים שבוצעו בתקופת המחקר

<u>מטרה מס' 1</u>: אנליזה גנומית משווה: קביעת מועדי איסוף, וביצוע האנליזה

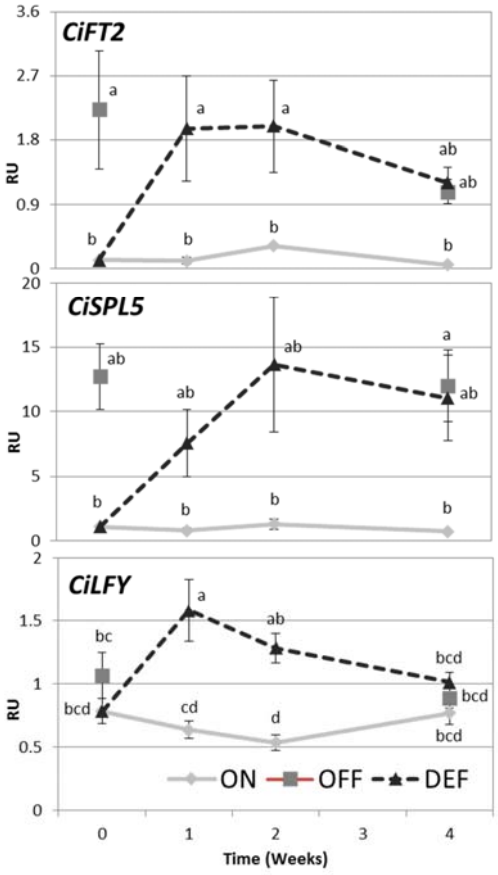
פרי הוסר לחלוטין מעצי מורקוט בשנת שפע בשליש האחרון של חודש אוגוסט, ופקעים נאספו לאחר שבוע, שבועיים וארבעה שבועות. ספירת פרחים באביב הבא הראתה את יעילות הדילול, לפחות לגבי פריחה טהורה (איור 1); אם בעצי שפע אחוז הליבלובים הטהורים היה כ-20, הרי בעצים מדוללים האחוז עלה לכ-70, בדומה לעצי שפל. מגמה הפוכה נצפתה לגבי לבלובים ווגטטיביים.

רנ"א מוצא, ועל מנת לבחון את יעילות הטיפול ומהירות התגובה של העץ, נבחנה רמת ה-mRNA של גנים שונים, אשר בעבודה הקודמת הראו שינויי בביטויים בפקעי שנת שפע לעומת פקעי שנת שפל. לקביעת מהירות התגובה הייתה חשיבות



איור 1: התפלגות סוגי התפרחות השונות באחוזים בעצי שפע (ON), עצי שפל (OFF) ועצי שפע מדוללי פרי (DEF). ממוצעים של 3 חזרות ביולוגיות, כולל טעויות תקן. האותיות מייצגות שונות סטטיסטית ($P \leq 0.05$) בין המצבים.

עבורנו בקביעת המועדים של האנליזה הגנומית. גנים אלו (כ-20 סה"כ) כללו גנים לפריחה, גנים למטבוליזם של טרהלוז וגנים ביוסינטתיים של פלבונואידים. מכלל הגנים שנבחנו ב-real time PCR או ב- nCounter analysis, אנו מציגים כאן תגובת שלשה גנים מבקרי פריחה אשר שניים מהם, *FT2* ו-*SPL5*, הראו ביטוי גבוה בפקעי עצי שנת שפל לעומת פקעי שנת שפע בתקופת השנה בה בוצע הניסוי (איור 2). כצפוי, שניהם מראים עליה בביטויים שבוע לאחר הדילול, והם מגיעים לרמת הביטוי בפקעי שנת שפל. מעניין שגם הגן *Leafy*, אשר בתקופה זו של השנה לא מראה שינויי בביטויו בין שני המצבים, הראה גם הוא עלייה בביטוי שבוע ושבועיים בפקעי עצים מדוללים, אך חזר לרמתו הנורמאלית אחרי ארבעה שבועות. כל הגנים האחרים שנבחנו הראו שינוי בביטויים תוך שבוע עד שבועיים מרגע הדילול, בהתאם לצפוי.

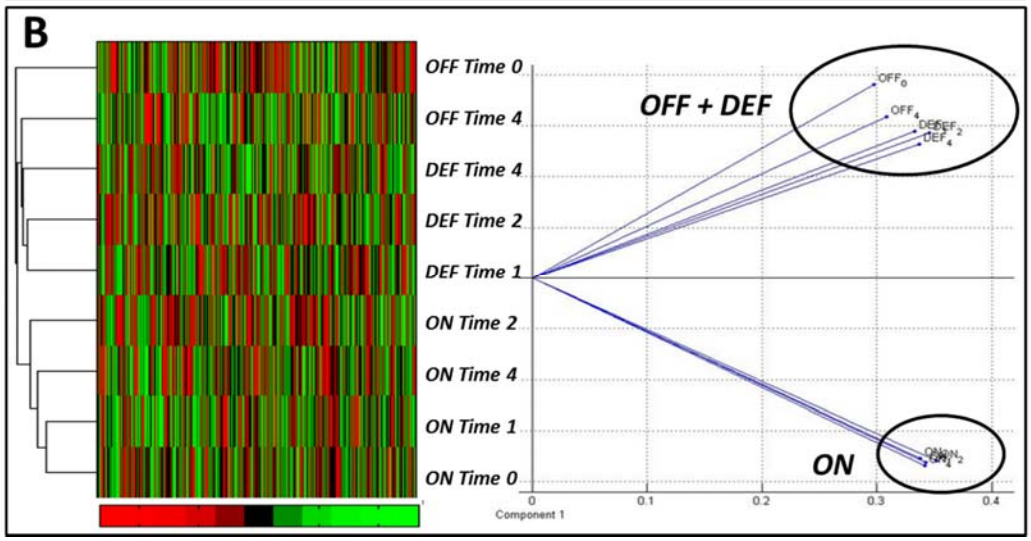


איור 2: ביטוי גנים לפריחה בפקעי עצי שפע (ON), עצי שפל (OFF) ועצי שפע מדוללי פרי (DEF) בזמן המצויין לאחר הדילול. ממוצעים של 3 חזרות ביולוגיות, כולל טעויות תקן. האותיות מייצגות שונות סטטיסטית ($P\leq0.05$) באותה נקודת זמן.

ריצוף עמוק בגישת illumina של רנ"א מפקעים לאחר דילול פרי בוצע ביחידת השירות של אוניברסיטת ת"א, והאנליזה הביואינפורמטית הבסיסית בוצעה בת"א, ובהמשך ע"י יחידת השירות של המכון למדעי הצמח. תיאור סכמתי של הניסוי ומועדי האיסוף מתוארים באיור 3A. אנליזות Hierarchical Clustering Heatmap (איור 3B, שמאל) ו- 2D Principle Component (איור 3B, ימין) הראו שכבר לאחר שבוע פקעי עצים מדוללים מראים דגם גלובאלי של ביטוי גנים דומה לזה של פקעי שנת שפל. תוצאות אלו מאששות את מהירות התגובה של העץ לדילול. עפ"י סכמת הניסוי (איור 3A) ניתן לראות כי רק בנקודת הזמן של ארבעה שבועות ניתן היה להשוות בין שלשת הטיפולים. בנקודת זמן זו בחנו כמה גנים משתנים בין פקעי שנת שפע לפקעי שנת שפל ($P\leq0.05$), והסתבר כי 997 גנים בפקעי שנת שפל ו-797 גנים בפקעי עצים מדוללים הראו ביטוי מופחת לעומת פקעי שנת שפע, כאשר 615 גנים היו משותפים בין שתי הקבוצות (איור 4A). סה"כ, 959 גנים בפקעי שפל ו-920 גנים בפקעי עצים מדוללים עלו בביטויים לעומת פקעי שפע, עם 564 גנים משותפים בין שתי הקבוצות. השוואה נוספת שבוצעה הייתה השוואה התפתחותית, ומטרתה זיהוי גנים שהראו שוני בביטויים בזמן הניסוי. לצורך כך בוצעה אנליזת אשכולות ביטוי (clustering analysis), כאשר ביטוי הגנים הושווה בכל נקודות הזמן לזמן 0. ארבעה אשכולות של גנים זוהו עבור פקעי שפע ושפל (איור 4B). מאחר ועבור פקעי שנת שפל האנליזה כללה רק שתי נקודות זמן, ניתן היה לזות גנים שעלו או ירדו

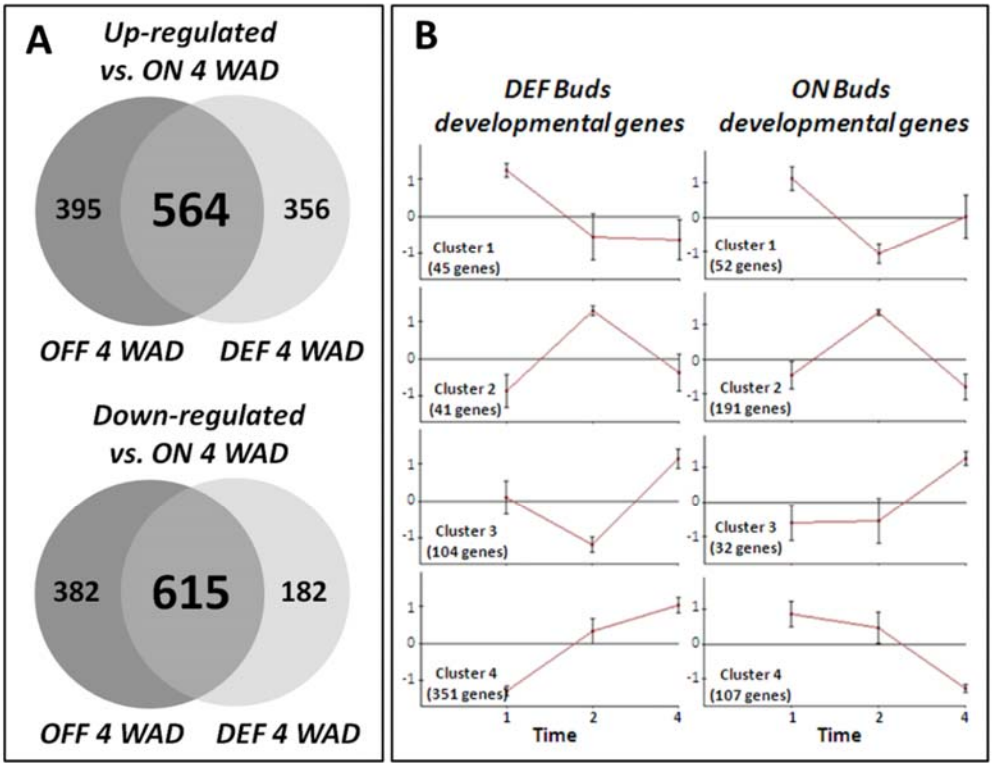
A

Tree status\Samples collection	Time 0	Time 1	Time 2	Time 4
ON	X	X	X	X
DEFRUITING (DEF)		X	X	X
OFF	X			X



איור 3: ביטוי גלובלי של גנים בפקעי עצים מדוללים (DEF) דומה לזה של עצי שפל (OFF) מאשר לזה של עצי שפע (ON). טבלת איסוף דגימות (A). משמאל, Hierarchical clustering heatmap ומימין, 2D principle component analysis (B). הגרפים יוצרו תוך שימוש בערך log FPKM-של כל גן בדגימות המצוינות ב-A.

במהלך תקופת הניסוי (תוצאות לא מוצגות). ע"מ לזהות תהליכים מטבוליים ובקרתיים המשתנים בפקעי שנת שפל ופקעי עצים מדוללים לעומת פקעי שפע, גנים שעלו או ירדו בביטויים עברו אנוטציות Gene Ontology (GO) ונותחו באמצעות אנליזת Singular Enrichment Analysis (SEA). מפאת קוצר מקום, אנו לא מציגים את כל התוצאות (מובאות במאמר המצורף).



איור 4: דיאגרמות Venn של גנים דיפרנציאליים ($P \leq 0.05$) המתבטאים בפקעי עצים מדוללים (DEF) ועצי שפל (OFF) ביחס לעצי שפע (ON) ארבעה שבועות לאחר הדילול (A). אנליזת אשכולות ביטוי (clustering) של גנים המשתנים התפתחותית ($P \leq 0.05$) בפקעי שנת שפע (ON) ובפקעי עצים מדוללים (DEF) במהלך הניסוי ביחס לנקודת זמן 0. האשכולות יוצרו ע"י אלגוריתם Click (http://acgt.cs.tau.ac.il/expander/overview.html).

תהליכים משותפים לפקעי שפל\עצים מדוללים שעלו יחסית לפקעי שפע כללו כאלו שהיו קשורים לחישת אור, פוטוסינתזה, ראורגניזציה של הכלורופלסט, תגובה לסוכרים, והומאוסטאזיז של יונים. גנים שירדו בפקעי שפל\עצים מדוללים יחסית לפקעי שפע מופו להרבה פחות תהליכים שכללו מטבוליזם שניוני, ותהליכי חימצון חיזור. האנליזות הנ"ל בוצעו גם לגנים שהתשנו בביטויים במהלך הניסוי, אולם ניתן היה למפות את clusters 3, 4 של פקעי עצים מדוללים וכן את cluster 2 של פקעי שפע, כאשר שאר ה-clusters לא מופו. תיאור של תהליכים אלו מופיע במאמר המצורף.

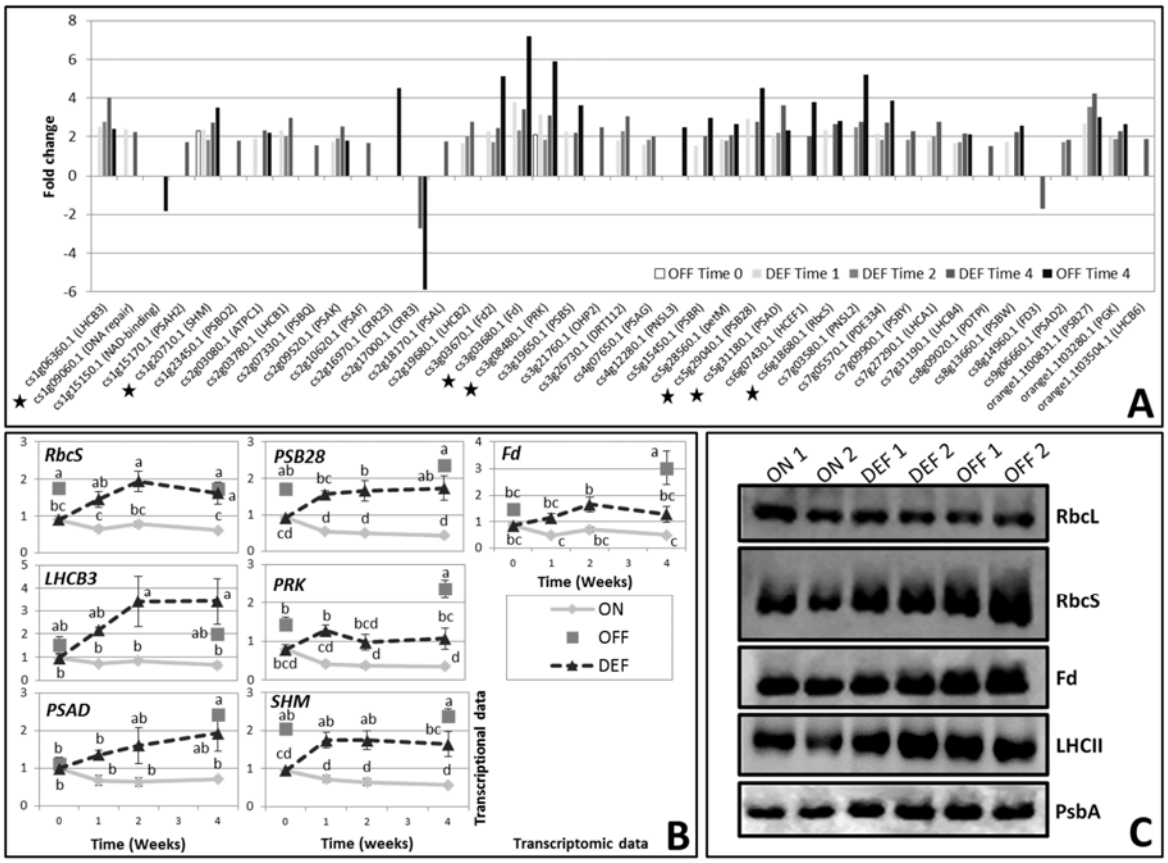
<u>מטרה מס' 2</u>: אימותים מולקולאריים וביוכימיים

אימותים מולקולאריים וביוכימיים בוצעו לשלשה תהליכים, פוטוסינתזה, מטבוליזם של חומצה אבסיסית (ABA) וטרנספורט פולארי-תלוי סידן של אוקסין.

שינויים בגנים לפוטוסינתזה

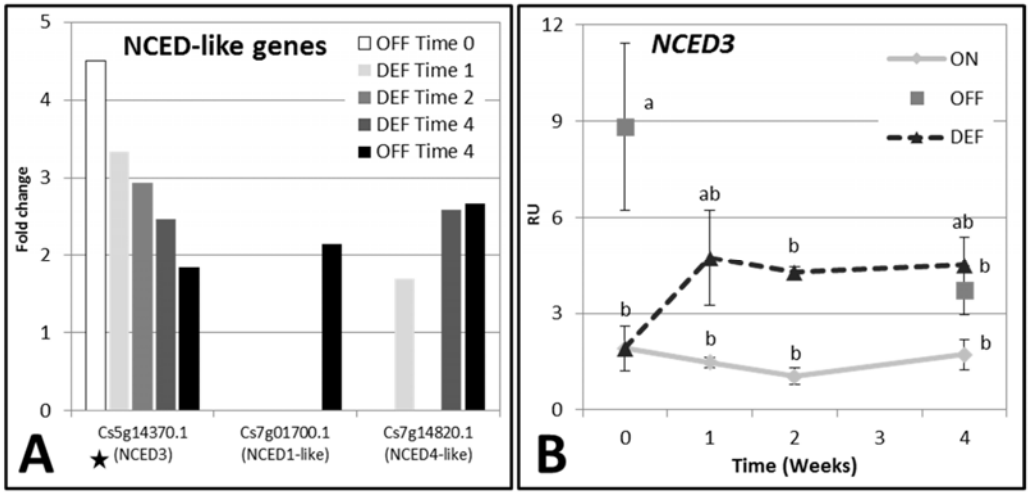
באנליזה הגנומית שנעשתה במסגרת התכנית הקודמת לפקעי שנת שפע ושפל במהלך חודש מאי, התברר כי יש עלייה משמעותית במרבית הגנים השייכים למערכת הפוטוסינטתית, על שלשת המסלולים שלה, ראקציית האור, מעגל קאלווין ופוטופוספורילציה. בתכנית הנוכחית חזרה תוצאה זו על עצמה. מתוך 41 גנים ששויכו למערכת הפוטוסינטתית, 38 הראו עליה כבר אחרי שבוע לאחר דילול פרי, וחלקם המשיך לעלות בביטויו עד תום הניסוי (איור 5). רק שלשה גנים הראו ירידה בביטויים לפחות באחת מנקודות הזמן שנבחנו. אימות ע"י real time PCR בוצע לשבעה גנים השייכים לאחת משלשת הריאקציות הנ"ל, *Ribulose bisphosphate carboxylase -small subunit* (*RbcS*), *Light-harvesting chlorophyll B-binding protein* (*LHCB3*), *Phosphoribulokinase* (*PRK*), *Photosystem II reaction center PSB28* (*PSB28*), *Photosystem I subunit D* (*PSAD*), *Serine hydroxymethyltransferase* (*SHM*), ו*Ferredoxin* (*Fd*). רמת הרנ"א של כל הגנים שנבחנו הייתה, כמצופה, גבוהה בפקעי שנת שפל לעומת פקעי שנת שפע. דילול פרי הביא לעליה בביטויים תוך שבוע בס"ג של פי 2.6 ועד 5.2. יתרה מזאת, שימוש בנוגדנים ספיציפיים כנגד RbcS, Fd, LHCII ו-PsbA הראה תמונה דומה לגבי רמת החלבון, היא הייתה גבוה בפקעי שפל לעומת פקעי שפע, ועלתה לאחר דילול. מעניין ש- RbcL יצא דופן, ולמרות העלייה ברמת הרנ"א, לא אובחנה עליה ברמת החלבון.

איור 5 (עמוד הבא): המערכת הפוטוסינתטית בפקעי שנת שפל ופקעי עצים מדוללים עולה. שוני בביטוי ($P\leq0.05$) של גנים פוטוסינטתיים שזוהו ע"י תכנת Mapman בפקעי עצים מדוללים (DEF) ופקעי עצי שפל (OFF) ביחס לפקעי עצי שפע באותה נקודת זמן (A). אנליזת real time PCR של מספר גנים במערכת הפוטוסינתתית, כמפורט בגוף הטקסט בפקעי עצים מדוללים (DEF), פקעי עצי שפע (ON) ושפל (OFF) (B). ממוצעים של 3 חזרות ביולוגיות עם טעיות תקן. האותיות מייצגות שוני סטטיסטי ($P\leq0.05$) באותה נקודת זמן בין המצבים. אנליזת Immunoblot לחלבונים המצוינים, כמפורט בגוף הטקסט, בשתי חזרות ביולוגיות בכל אחד מהמצבים המפורטים לעיל (C).



מטבוליזם של חומצה אבסיסית (ABA)

תוצאות האנליזה הגנומית הראו כי גנים הומולוגים ל- *9-cis-epoxycarotenoid dioxygenase* (*NCED*), המקודדים לשלב קובע המהירות של ביוסינתזה של ABA גבוהים ברמת הרנ"א שלהם בפקעי שפל לעומת פקעי שפע, ועולים לאחר דילול. בארבידופסיס משפחה זו כוללת תשעה גנים, והוכחה ישירה למעורבות ארבעה מהם, *NCED 2, 3, 5, 6*, בביוסינתזה של ההורמון קיימת. גנום ההדרים כולל תשעה גנים הומולוגיים, ושינויים בביטוי באנליזה הגנומית אובחנו בשלשה מהם, אחד

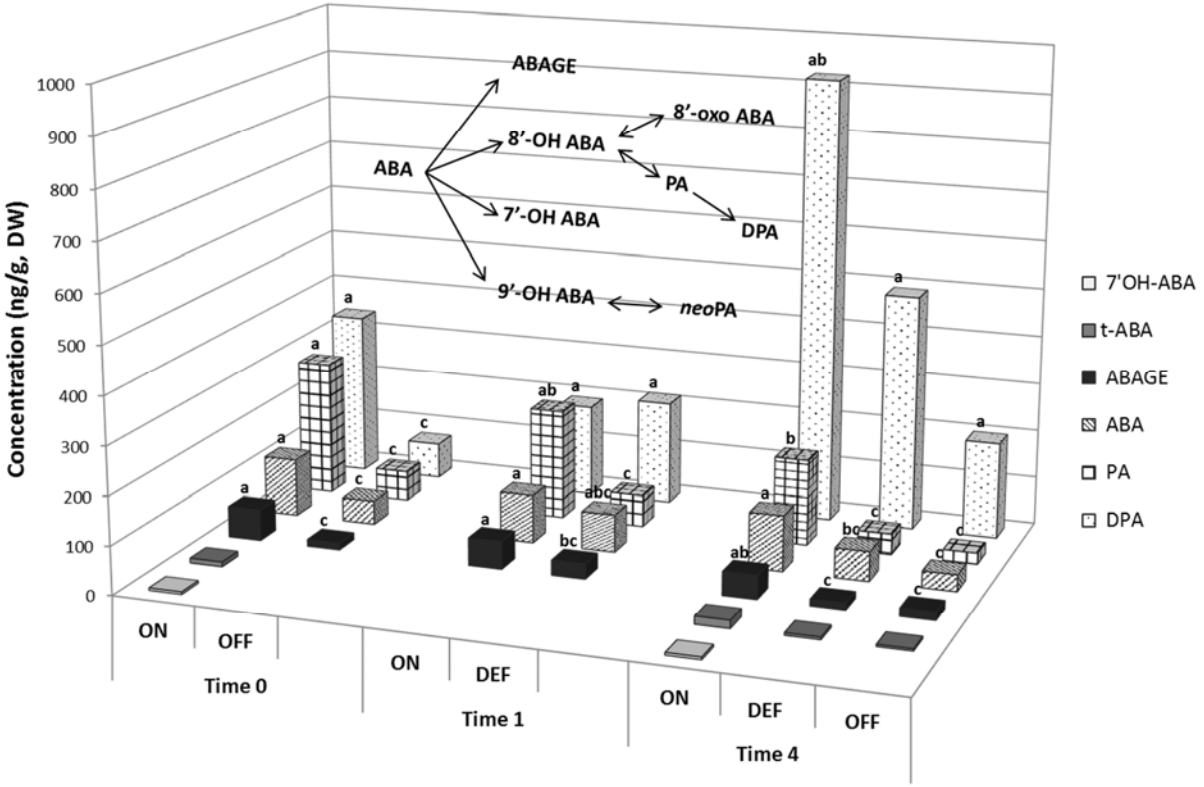


איור 6: עליה בביטוי גנים לביוסינתזה של ABA. עליה בביטוי גנים לביוסינתזה של ABA באנליזה הגנומית ($P\leq0.05$) בפקעי עצים מדוללים (DEF) ועצי שפל (OFF) ביחס לעצי שפע (ON) באותה נקודת זמן (A). ביטוי הגן NCED3 במצבים הנ"ל בזמנים שונים לאחר הדילול כפי שאופיין ב- real time PCR (B). ממוצע שלש חזרות ביולוגיות עם טעויות תקן. האותיות מייצגות שונות סטטיסטית ($P\leq0.05$).

הומולוגי ל-*NCED3* מארבידופסיס, אחד הומולוגי ל-*NCED1*, ואחד הומולוגי ל-*NCED4* (איור 6A). זה שהראה הומולוגיה ל-*NCED3* הראה עליה ברורה בביטויו בכל נקודות הזמן בין פקעי שנת שפל\עצים מדוללים לפקעי שנת שפע, והוא אומת באנליזת Real Time PCR. שני הגנים האחרים הראו עליה רק בחלק מנקודות הזמן של הניסוי.

תוצאה זו הביאה אותנו לבצע אנליזה של רמת ההורמון והקטבוליטים שלו בזמן 0, לאחר שבוע (פקעי שפע ופקעי עצים מדוללים), ולאחר ארבעה שבועות בכל אחד מהטיפולים (איור 7). מסלול הקטבוליזם של ABA מופיע בחלקו הפנימי של איור 7. להפתעתנו, למרות העליה ברמת הגן הביוסינתתי של ההורמון, רמת ה-ABA הייתה נמוכה בפקעי שפל לעומת פקעי שפע בכל נקודות הזמן שנבחנו, כאשר בפקעי עצים מדוללים אובחנה ירידה כבר לאחר שבוע. גם קטבוליטים שונים של ההורמון, ABAGE ו-PA, הראו ירידה ברמתם, וכן אובחנה ירידה באיזומר של ההורמון t-ABA.

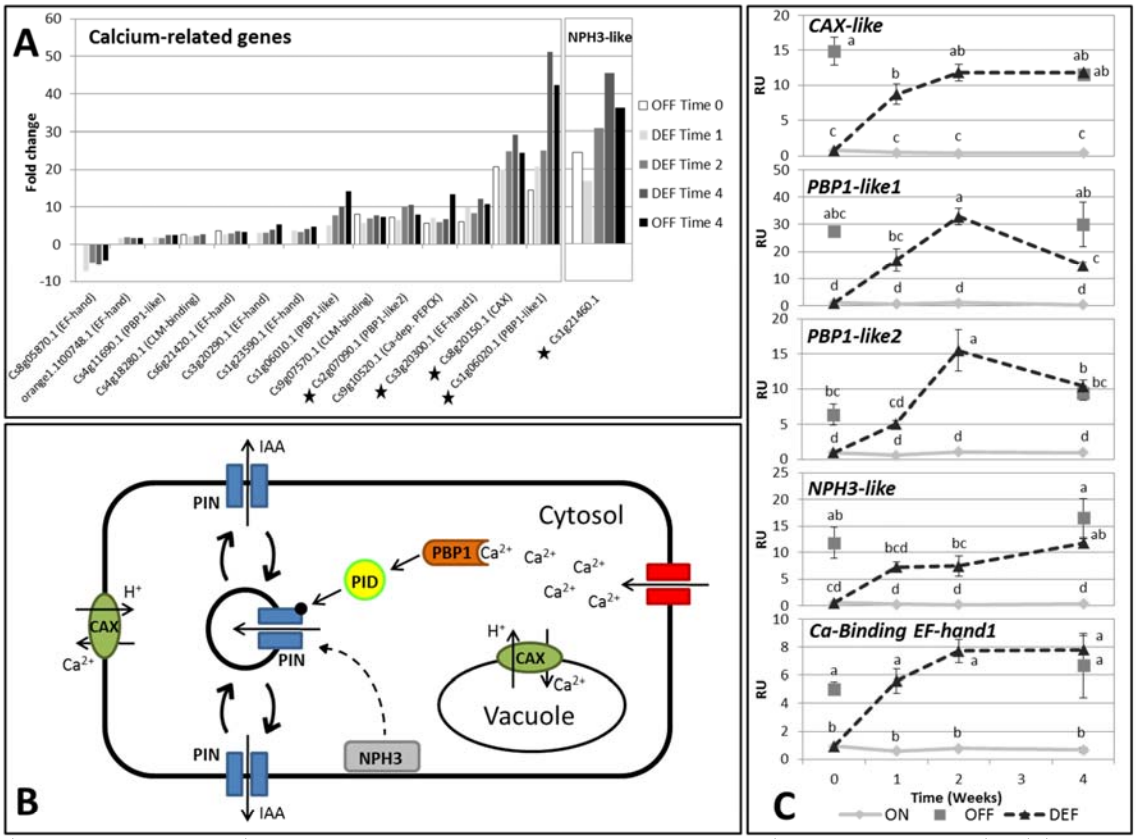
איור 7: דילול מוריד רמת ההורמון ABA וקטבוליטים שלו. רמת ההורמון וקטבוליטים שלו נבחנה בפקעי עצים מדוללים (DEF), עצי שפל (OFF) ולעצי שפע (ON) בנקודות זמן לאחר הדילול, כמצוין. ממוצע שלש חזרות ביולוגיות עם טעויות תקן. האותיות מייצגות שונות סטטיסטית ($P \leq 0.05$). הסכימה במרכז מציגה תיאור סכמתי של מסלול הקטבוליזם של ההורמון.



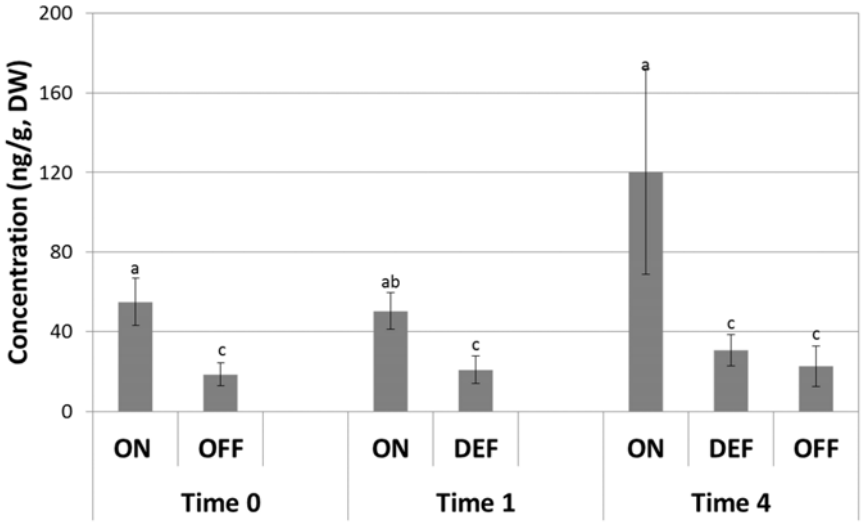
טרנספורט פולארי תלוי-סידן של אוקסין

בין הגנים שהראו עליה גבוהה יחסית בביטויים, פי 3 עד פי 40, בפקעי שנת שפל\עצים מדוללים לעומת פקעי שנת שפע היו גנים הקשורים לסידן. רבים מגנים אלו מקודדים לחלבונים המכילים אתר הקרוי EF-hand, כולל חלבון הקרוי PINOID-binding protein (PBP)-(PIN), אחד מהם, PBP1 משחק תפקיד בטרנספורט פולארי של אוקסין כתלות ברמת הסידן בתא. ארבעה גנים דומים (מתוך חמישה המצויים בגנום ההדר) עלו בביטויים בפקעי שנת שפל\עצים מדוללים לעומת פקעי שפע. גנים

נוספים הקשורים לסידן שעלו בביטויים בדרך דומה כללו שניים הומולוגים ל-Ca-dependent protein kinase ול-Ca/Proton antiporter (CAX) (איור 8A). גן נוסף שעלה בביטויו הראה הומולוגיה לגנים ממשחת ה-*NPH3*, שמשחקים גם הם תפקדי בטרנספורט פולארי של אוקסין, כנראה ללא תלות בסידן. הקשרים הבקרתיים בין סידן תאי פעילות כל הגנים הללו וטרנספורט פולארי של אוקסין מוצגים באיור 8B. הביטוי של CAX-like, NPH3-like, שני PBP1-like, וגן הדומה ל-Ca-binding-EF-Hand אומתו בעזרת Real-Time PCR. (איור 8C). הביטוי של כולם היה נמוך בפקעי שנת שפע לאורך כל תקופת הניסוי, אולם היה גבוה משמעותית בפקעי שנת שפל. כצפוי, דילול הביא לעליה משמעותית בביטויים כבר לאחר שבוע. תוצאות אלו הביאו אותנו לבחון את רמת האוקסין בפקעים בזמן 0, ובשתי נקודות זמן נוספות (איור 9). רמת ההורמון הייתה גבוה יחסית בפקעי שנת שפע לעומת פקעי שנת שפל, וכצפוי היא ירדה לאחר דילול פרי.



איור 8: דילול מעלה ביטוי גנים הקשורים לסידן תוך תאי וכן את הגן NPH3 בפקעים. שוני בביטוי באנליזה הגנומית ($P\leq0.05$) של גנים הקשורים להומאוסטזיז של סידן ו-NPH3 בפקעי עצים מדוללים (DEF) ופקעי עצי שפל (OFF) ביחס לפקעי עצי שפע באותה נקודת זמן (A). היחסים הבקרתיים בין החלבונים השונים הקושרים בין הומאוסטאזיז של סידן לולקליציה ופעילות של חלבוני PIN, האחראיים על טרנספורט פולרי של אוקסין (B). ביטוי הגנים המצוינים נבחן באנליזת real time PCR בפקעי עצים מדוללים (DEF), עצי שפל (OFF) ולעצי שפע (ON) בנקודות זמן לאחר הדילול, כמצוין (C). ממוצע שלש חזרות ביולוגיות עם טעויות תקן. האותיות מייצגות שונות סטטיסטית ($P\leq0.05$).



איור 9: דילול מוריד רמת ההורמון אוקסין בפקעים. רמת ההורמון החופשי נבחנה בפקעי עצים מדוללים (DEF), עצי שפל (OFF) ולעצי שפע (ON) בנקודות זמן לאחר הדילול, כמצוין. ממוצע שלש חזרות ביולוגיות עם טעויות תקן. האותיות מייצגות שונות סטטיסטית ($P \leq 0.05$).

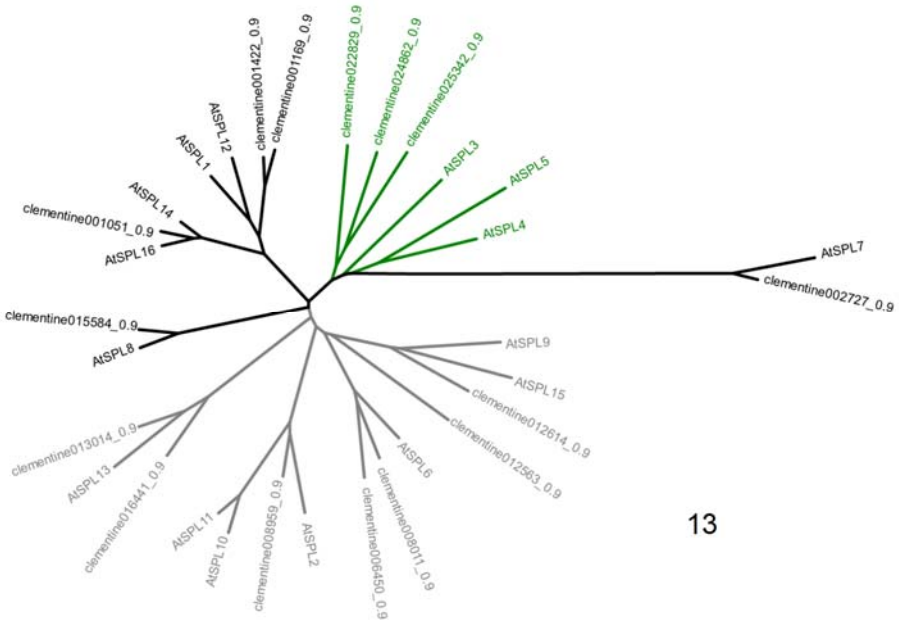
<u>מטרה מס' 3</u>: הגן *SPL5* וסירוגיות

כאמור, אחד הממצאים מהתכנית הקודמת היה הגילוי כי גן הומולוגי לגנים ממשפחת ה-*SPL* מארבידופסיס עולה בביטויו באברי עצי שפל לעומת עצי שפע בכל נקודות הזמן שנבחנו. גנים ממשפחה זו הנם בקרי-על לפריחה; הם מבקרים גנים לפריחה כמו FT ו-Leafy, ולמעשה אחראים על מעבר פאזות מצמח יובנילי לבוגר וכן על פריחה עונתית. תפקידם החשוב הביא אותנו להעמיק במחקר עליהם, ולנסות לקשור פעילותם להשפעת עומס הפרי על הפריחה.

אנליזה ביואינפורמטית

משפחת ה-*SPL* כוללת מספר גנים בגנום הארבידופסיס, שלהם הומולוגיים בגנום ההדר (איור 10). ההתפצלות בין הגנים השונים חלה לאחר ההתפצלות בין הדרים לארבידופסיס. חלק מהגנים הנם יחסית ארוכים, וחלקם, *SPL3,4,5* הנם גנים קצרים המאופיינים באתר קישור ל-*miR156* ב -3'-UTR שלהם ומבוקרים על ידו. הגן שזיהינו (*Clemetine025342_0.9*) הנו אחד

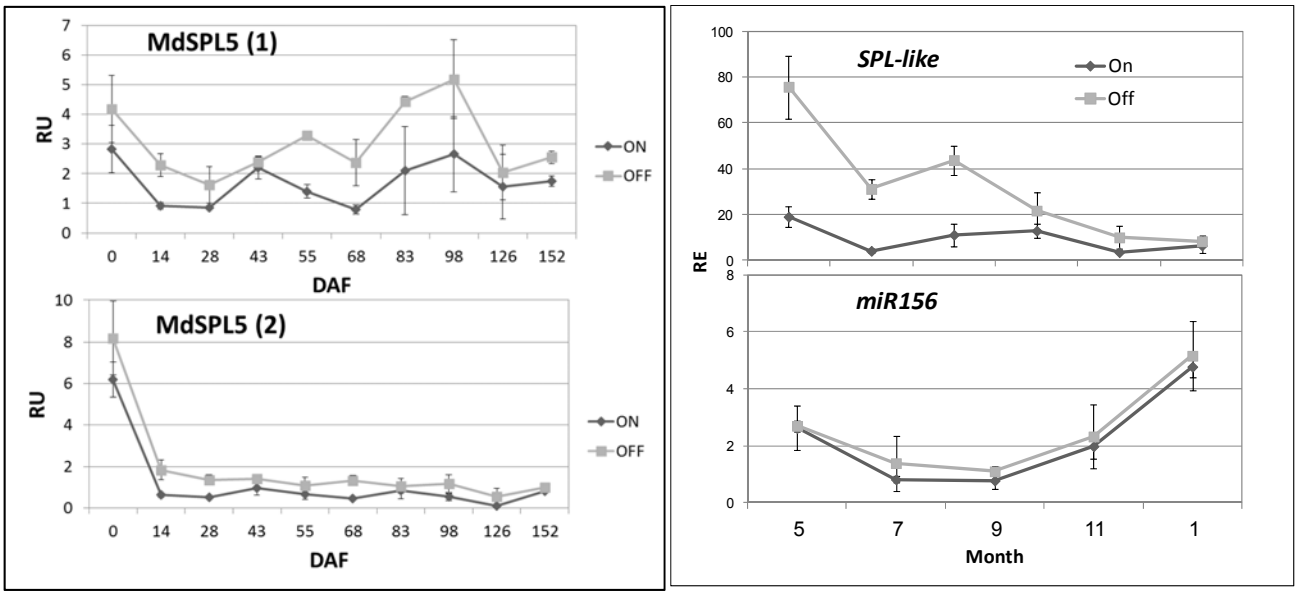
איור 10: עץ פילוגני של גנים ממשפחת ה-*SPL* בהדרים (קידומת clementine) ובארבידופסיס (קידומת At). בירוק, גנים ממשפחת ה- *SPL* הקצרים (3, 4, 5).



משלשה גנים קצרים, אשר מצויים על אותו הענף יחד עם *SPL3,4,5* מארבידופסיס, וחולקים עמם הומולוגיה גבוהה יחסית. לשלשתם אתר כמעט זהה לאתר הקישור של *miR156* מארבידופסיס. על סמך דמיון באזור גנומי שסביב הגן, כפי שיתואר בהמשך, הגן אותו אפיינו מהדרים הנו כפי הנראה *SPL5* .

ביטוי הגן בהדרים ובתפוח

כאמור, הגן *SPL* מבוקר ע"י *miR156*. מכאן שצפוי (אך לא הכרחי) לקבל ביטוי רציפרוקלי של ביטוי הגן לעומת ביטוי ה-mIR. ביטוי הגן *SPL* נבחן מחודש מאי ועד חודש ינואר באמצעות Real Time PCR. כצפוי, פקעי שנת שפל הראו ביטוי גבוה לאורך רוב התקופה מפקעי שנת שפע. כללית, שיא הביטוי בפקעי שפל היה בחודש מאי, והוא ירד בהדרגה, עם פיק בספטמבר (איור 11 ימין). כבר הצגנו תוצאה זו בתוכנית המחקר, ועמדנו על הדמיון בביטוי גן זה לביטוי הגנים *Leafy* ו-*AP1* המבוקרים ע"י *SPL*. בחינת ביטוי *miR156* באותן דגימות הראתה כי לא היה הבדל בביטוי בין פקעי שנת שפע לפקעי שנת שפל, אולם באופן כללי, דגם הביטוי היה בקירוב רציפרוקלי לזה של *SPL*, כלומר הוא עלה במהלך התקופה הנבחנת עם הירידה בביטוי הגן *SPL*. בגנום התפוח זוהו שני גנים דומים לגן SPL5. רנ"א התקבל באדיבות ד"ר אלון סמך, והוא מוצה בזמנים שונים לאחר פריחה מדורבנות של עצי שפע מהם הוסרו פרחים וחנטים, וכן מעצי ביקורת. מתוך שני הגנים שנבחנו, רק (11, שמאל). נראה שלקראת תקופת האינדוקציה לפריחה, המתרחשת כ-100 יום לאחר שיא פריחה, ביטוי הגן היה הגבוה ביותר, ובתקופה שקדמה לה, נמדדו ההבדלים הגדולים ביותר בין שני המצבים.



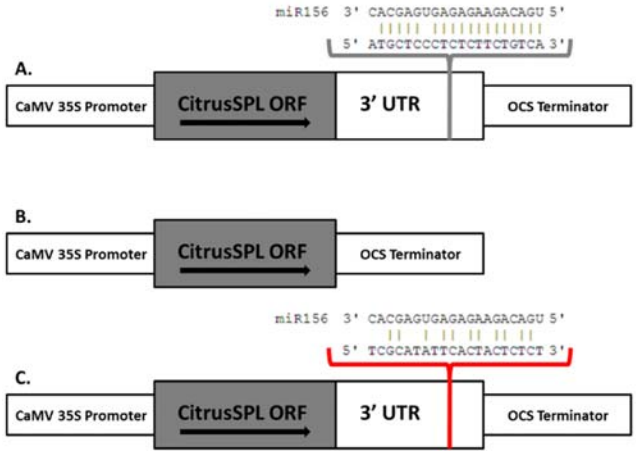
איור 11: ביטוי הגן *SPL* בהדרים ובתפוח. ביטוי הגן ו-*miR1565* בהדרים (ימין) בוצע בפקעי עצי שפל (OFF) ושפע (ON) בחודשים המצוינים. ביטוי שני גנים הומולוגים בעלי תפוח מדוללי פרחים ופרי (OFF) או לא מדוללים (ON) בימים המצוינים לאחר פריחה (DAF) (שמאל). ממוצעים של 3 חזרות ביולוגיות עם טעויות תקן.

בחינה פונקציונאלית של תוצר הגן מהדרים

על מנת להוכיח שתוצר הגן בהדרים אכן בעל פעילות ביולוגית הקשורה לבקרת פריחה, בחנו פעילותו בצמחי ארבידופסיס. הוכנו הקונסטרקטים הבאים (איור 12):

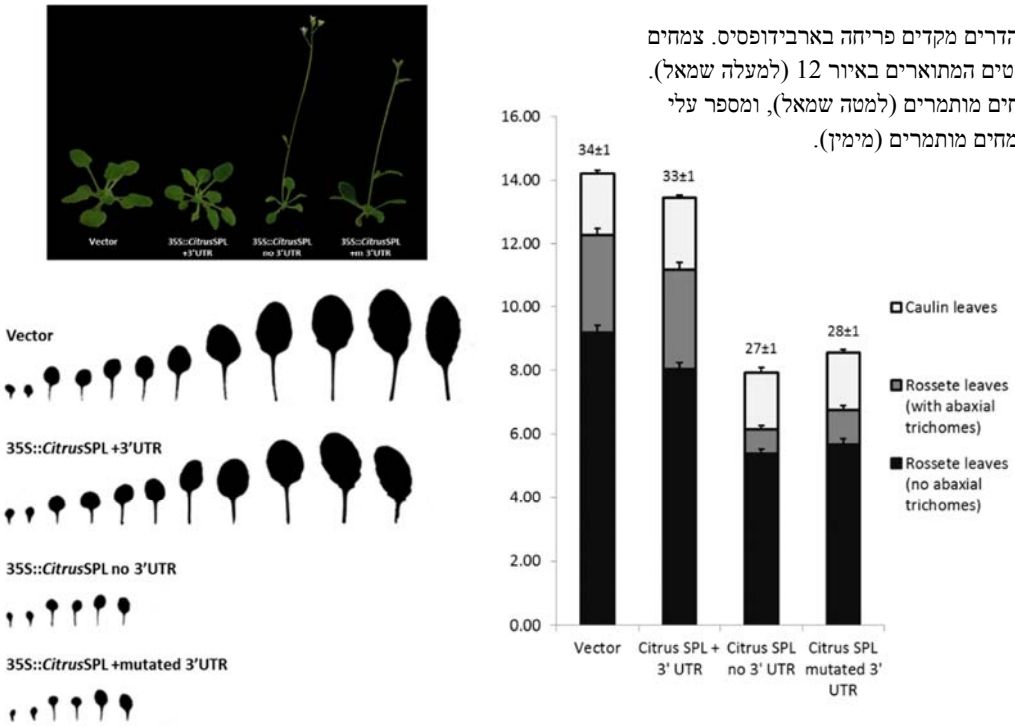
1. האזור המקודד ל-*SPL3-like* צמוד ל-3'UTR נורמאלי של הגן.
2. האזור המקודד ל-*SPL3-like* צמוד ל-3'UTR בו אתר הקישור ל-miR156 פגוע.
3. האזור המקודד ל-*SPL3-like* ללא 3'-UTR.

איור 12: תיאור סכמתי של הקונסטרקטים ששמשו להתמרת ארבידופסיס והדרים. קונסטרקט על אתר miR156 לא מופרע (A).קונסטרקט ללא 3'-UTR (B) . קונסטרקט עם 3'-UTR בו אתר הקישור של miR156 עבר מוטגנזה מכוונת (C).



האזורים המקודדים הוצמדו ל-35S promoter, והם הוחדרו לאגרובקטריום ובשיטת טבילת פרחים, לארבידופסיס. הפנוטיפ של הצמחים נבחן ביחס לזה של זן הבר (איור 13). כצפוי, כאשר ה-ORF לא הכיל את ה-3`UTR המכיל את אתר הקישור ל-miR156, או כאשר אתר הקישור עבר מוטגנזה, התקבלה פריחה מוקדמת לעומת זן הבר, זן הבר אליו הוחדר וקטור בלבד

איור 13: הגן *SPL5* מהדרים מקדים פריחה בארבידופסיס. צמחים מותמרים עם הקונסטרקטים המתוארים באיור 12 (למעלה שמאל). צילום של עלים של צמחים מותמרים (למטה שמאל), ומספר עלי Rosette ו-Caulin בצמחים מותמרים (מימין).



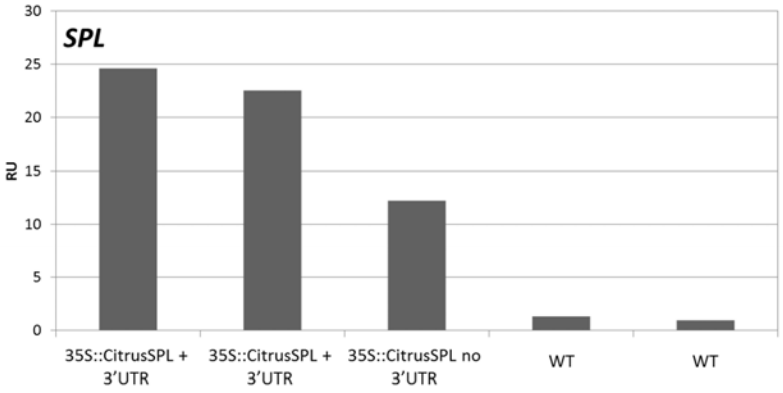
או צמח אליו הוחדר ה-ORF בצמוד ל-3`-UTR לא מופרע. מופע הקדמת הפריחה בא לידי ביטוי גם בפנוטיפים אחרים,

הקטנת מספר עלי הרוזטה ועלי הקאולין שהופיעו עד לפריחה. אין ספק כי ההוכחה האולטימטיבית לכך ש-*SPL5* מעורב בבקרת הפריחה החוזרת בסירוגיות צריכה להגיע מביטויו ביתר או מהפחתת ביטויו בהדרים.

Line	# of regenerated plants	# of Real transformants
35S::*Citrus*SPL + 3'UTR	10	10
35S::*Citrus*SPL no 3'UTR	6	1
35S::*Citrus*SPL + mutated 3'UTR	2	0

איור 14: צמחי הדרים מותמרים עם קונסטרקטים שונים המתוארים באיור 12 (למעלה). מספר צמחים רגנרנטיים ומותמרים בפועל מכל קונסטראקט (למטה).

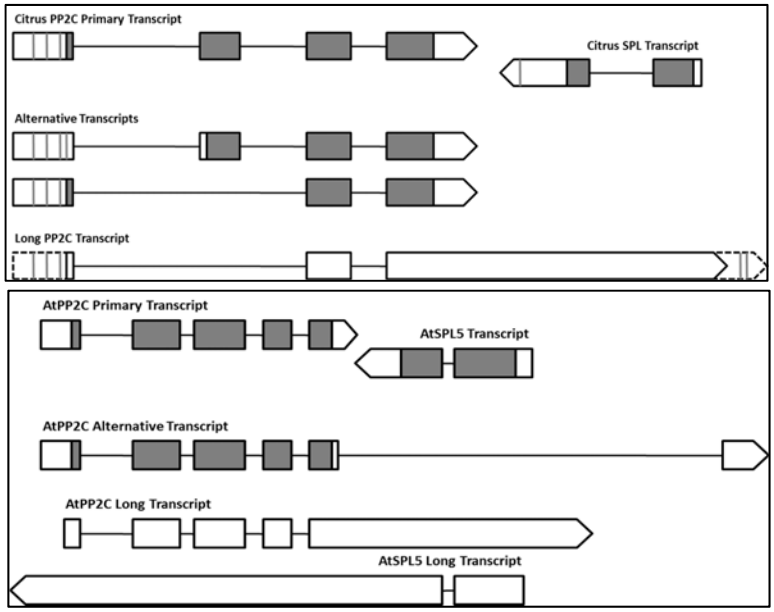
הקונסטרקטים הנ"ל הותמרו לזן הדרים טרוייר. למרות שקיבלנו רגנרנטיים משלשת הקונסטרקטיים, בפועל, בחינה מדוקדקת שלהם, הראתה כי בעוד שעשרה הרגנרטניים של הקונסטראקט מס' 1, לעיל, היו אכן טרנסגניים, רק טרנסגן אחד התקבל מקונסטרקט מס' 2, ואף לא אחד מקונסטראקט מס' 3 (איור 14). בחינת ביטוי של הגן בצמחים הטרנסגניים שהתקבלו הראתה כי בכולם רמת ביטוי הגן הייתה גבוה מאשר בצמחי ביקורת, כולל הצמחים שהכילו 3-UTR לא מופרע שאר אמור לקשור miR156, מה שמצביע על כך שהגן אולי יוצר בעודף לעומת ה-mIR156 (איור 15). כך או כך, נכון למועד כתיבת דו"ח זה, הצמחים עדיין לא מראים פנוטיפ של הקדמת פריחה, גם כאשר הודגרו בתנאים אינדוקטיביים לפריחה.



איור 15: ביטוי הגן *SPL5* בקווים של צמחי הדרים מותמרים בקונסטרקטים המתוארים באיור 12. רמת הביטוי נבחנה ב-real time PCR בצמחים אינדיבידואלים שהוכחו כמותמרים (איור 14)

אנליזה של האזור הגנומי של *SPL5*, ובחינת מספר התעתיקים ממנו

כאשר בחנו תוצרי שיעתוק של הגן *SPL5* מהדרים, גילינו כי עשויים להיות מספר תעתיקים. אנליזה של האזור הגנומי בהדרים ובארבידופסיס גילתה מצב מעניין; בצמוד לגן, ובכיוון ההפוך קיים גן ממשפחת Protein phosphatase 2C (PP2C) (איור 16). זוהו תעתיקים שמתחילים בגן זה וכוללים גם את הרצף ההופכי של *SPL5*. PP2C הנו חלק ממשפחה גדולה של serine/threonine phosphatases, שחלק מהם מעורבים במעבר סיגנל של ההורמון ABA. ממצא



איור 16: האתר הגנומי של הגן *SPL5* בהדרים (למעלה) ובארבידופסיס (למטה) עם התעתיקים שאופיינו ממנו.

זה העלה את ההיפותזה שיש קשר בקרתי ופונקציונאלי בין ביטוי הגן PP2C לביטוי SPL5. נקטנו במספר גישות ע"מ לבחון היפותזה זו בהדרים ובארבידופסיס. מאחר ולא הצלחנו להוכיח היפותזה זו, אנו לא מציגים את התוצאות. להלן עיקרי הניסויים:

1. בחינת ביטוי תעתיק ארוך, הכולל את רצף *SPL*, וכלל התעתיקים של *PP2C* בפקעי שנת שפל ושפע ולאחר דילול פרי. לא היה הבדל בביטוי התעתיקים. מעניין שלאחר דילול פרי, הייתה ירידה בביטוי כלל התעתיקים, אבל לא בתעתיק הארוך. בחנו גם ביטוי תעתיקי *PP2C* בנבטים, עלים יובנילים, עלים בוגרים ותפרחות של ארבידופסיס, חלק מהתעתיקים הראו הבדלים בביטוי בנבטים, עליים יובנילייים ועלים בוגרים, אבל לא בתפרחות. בחינה של ביטוי תעתיק ארוך וכלל התעתיקי מהכיוון של *SPL5* באיברים הנ"ל של ארבידופסיס לא העלתה הבדלים בולטים.
2. מאחר ובמאגרי הזרעים של ארבידופסיס לא היה קיים מוטאנט בגן *PP2C*, יצרנו צמחים בהם ביטוי הגן הוגבר ע"י שימוש בפרומוטר 35S, וצמחים בהם ביטוי הגן הוקפחת ע"י שימוש ב-RNAi. לצמחים אלו לא היה פנוטיפ מבחינת פריחה.
3. לאחרונה, אופיין מנגנון בקרת ביטוי גנים בבע"ח ובשמרים, הקרוי Nonsense-mediated Decay (NMD). מנגנון זה מזהה תעתיקים לקויים המכילים קודון פסק מוקדם, ומשמיד אותם. בפועל מנגנון זה משחק תפקיד בבקרה על ביטוי גנים, כאשר 5-10% מהטרנסקריפטום משתנה בתאים פגומים ב-NMD. בארבידופסיס התברר כי במוטנטים בגנים *UPF3* ו-*UPF1* יש הצטברות של non-coding mRNAs, הכוללים גם תעתיקים הופכיים לתעתיקים מקודדים. אפיון מנגנון זה בצמחים עדיין בחיתוליו. כך או כך, היה מעניין לבחון האם ביטוי תעתיקי *SPL5* ו-*PP2C* ישתנה במוטנטים אלו של

ארבידופסיס. בפועל, בחינת מוטנטים אלו לא הניבה תוצאות מעניינות, לא מבחינת פנוטיפ פריחה או מבחינת הצטברות תעתיקים מהאזור הרלוונטי.

דיון

תקופת האינדוקציה לפריחה, שנקבעה לפני שנים רבות באמצעים הורטיקולטריים, מתחילה בהדרים באמצע נובמבר ונמשכת לרוב עד סוף דצמבר או תחילת ינואר (Monselise and Halevy 1964). ביטוי של גנים מבקרי פריחה מאשש כי זוהי אכן התקופה (Goldberg-Moeller et al. 2013; Munoz-Fambuena et al. 2011; 2012; Nishikawa et al. 2007; Shalom et al. 2012). ממצאים ניסויים מלמדים כי טמפרטורה נמוכה הנה הגורם האינדוקטיבי המרכזי, כאשר לקיצור תקופת האור יש השפעה מועטה, אם בכלל. עומס הפרי בזנים סירוגיים מהווה גורם חשוב המשפיע על האינדוקציה לפרי, וניתן להניח כי הוא משפיע על מוכנות הפקע לקבל את סיגנל הפריחה (Albrigo and Galán-Saúco 2004; Verreynne and Lovatt 2009). העבודה שבוצעה במסגרת התכנית הקודמת הראתה שהפקע "חש" את מידת עומס היבול כבר בחודש מאי, מיד לאחר הפריחה והחנטה, שכן הבדלים בביטוי גלובאלי של גנים ניכרים בין פקעי שפל ושפע כבר בתקופה זו (Shalom et al. 2012). מובן כי מצב הפקע אינו מקובע, שכן הסרת פרי עד לתקופת האינדוקציה, ואף במהלכה, יכולה להפוך פקע שפע לפקע שפל. אחת ממטרות הניסויים המתוארים הייתה לבחון מה מתרחש בפקע העץ המדולל בזמן המעבר שלו בין שני המצבים. מפתיע היה לגלות כמה מהר, תוך שבוע, השינויים מתרחשים, לפחות בהתבסס על שינויים בביטוי גנים. יתכן והשינוי הוא אף מהיר יותר אולם לו"ז הניסוי אינו מאפשר לגלות זאת. עפ"י מספר הפרחים שנספרו באביב שלאחר הסרת הפרי, הושוו במהלך הניסוי הגנומי שתי אוכלוסיות פקעים, אחת עם 96% והשנייה עם 55% סיכוי לפריחה. לכאורה, לא מדובר בהבדלים דרמטיים, אבל למרות זאת ניתן היה לזהות תהליכים חשובים, גם מטבוליים וגם בקרתיים המשתנים בין פקעי שנת שפע לפקעי שנת שפל. בסיס ההצלחה לניסוי הנוכחי, שבא לידי ביטוי בזיהוי תהליכים רגולטוריים, היה השימוש בריצוף עמוק של רנ"א. באנליזה הקודמת ההשוואה הגנומית התבססה על שימוש בצ'יפ Affymetrix, שהנו, מטבע הדברים, מוגבל לגנים המצויים עליו. אכן, לאחר ניצוח התוצאות של האנליזה הנוכחית, חזרנו ובדקנו האם "פספסנו" גנים חשובים באנליזה הקודמת. הסתבר כי לגנים אלו, כמו גנים המגיבים לרמת הסידן התאי, לא היה ייצוג ע"ג הצ'יפ. למעשה, בהתבסס על נתונים אחדים, אנו מעריכים רק 30-35% מהגנים שזוהו באנליזה הנוכחית, מצויים על הצ'יפ.

תוצאות המחקר עד כה מאששות תוצאות קודמות כי בפקע שנת השפל, ובפקע העובר למצב "שפל" יש עלייה משמעותית בביטוי גנים פוטוסינטתים. אנו מראים כאן כי עלייה זו מלווה גם בעלייה רמת החלבון, לפחות לגבי חלק מהגנים שנבחנו (Shalom et al. 2012). למרות שיש עבודות המראות כי אין הבדל בין פוטוסינתזה של עלי שפל לעלי שפע, מרבית העבודות מראות כי בעץ שפע יש עליה בפוטוסינתזה לעומת עץ השפל, ומטרתה אספקה מוגברת של מוטמעים לפרי המתפתח (Dejong 1986; Fujii and Kennedy 1985; Gucci et al. 1995; Iglesias et al. 2002; Monerri et al. 2011; Nebauer et al. 2013; Palmer et al. 1997; Roper et al. 1988; Syvertsen et al. 2003; Urban et al. 2004). במידה והשינויים שאובחנו מצביעים על עליה בפוטוסינתזה של הפקע, זה נראה הפוך למצב בעלים. רמת המוטמעים בעצי

השפל הנה גבוהה מאוד. לפקע, כמבלע, אין תחרות עם הפרי. מדוע תעלה בו הפוטוסינתזה, כאשר אין הוא סובל ממחסור במוטמעים? יתכן ולשינויים אלו תפקיד בקרתי כל שהוא; אולי הפקע מסמן על מצבו התזונתי ע"י שינויים אלו, חוסם זרימת עודפי מוטמעים אליו, וע"י כך משחק תפקיד עצמי בבקרת גורלו.

אחת התוצאות המעניינות שהתקבלו הייתה עליה בגנים המרכזיים לביוסינטזה של ABA בפקעי עצי שפל ובפקעי עצים מדוללים, אשר לוותה בירידה ברמת ההורמון והקטבוליטים שלו, וכן בירידה בביטוי של גן לרצפטור שלו, *PYR1-like* (תוצאה לא הוצגה). אחד ההסברים לסתירה זו יכולה לנבוע מכך שבפקעי שפע, מקור ה-ABA האנדוגני אינו בפקע עצמו אלא מחוץ לו, ותלוי בנוכחות פרי. ברגע שהפרי מוסר, יש ירידה ברמת ההורמון בפקע מתחת לסף רצוי מסוים, וזה עושה אינדוקציה ליצירתו ע"י שפעול המערכת הביוסינטתית שלו. מצד שני, אין לשלול בשלב זה ביטוי "עקר" של הגנים. מהספרות ידוע כי רמת ההורמון ABA והאיזומר שלו, tABA, אכן גבוהה בפקעי שנת שפע (Goldschmidt 1984; Jones et al. 1976). ייתכן ורמת הורמון גבוהה מצביע על עקה בה שרוי פקע השפע. יתכן וההורמון משחק תפקיד בדיכוי פריחה חוזרת, אם כי הממצאים מהספרות סותרים (Garcialuis et al. 1986; Koshita et al. 1999; Okuda 2000). יתכן וההורמון פעיל גם בשמירה על הפקע במצב לא פעיל, במיוחד בשנת השפע (Horvath et al. 2003; Little and Edit 1968; Shalom et al. 2012).

העלייה המשמעותית בביטוי גנים המגיבים ומשחקים תפקיד בהומאוסטאזיז של סידן תוך תאי, יחד עם עליה בביטוי גנים המשחקים תפקיד בטרנספורט פולארי של אוקסין (גם כזה שאינו תלוי בסידן תוך תאי, *NPH3*), כיוונה לכך שרמת האוקסין משתנה בפקע שנת השפל, ולאחר דילול (Benjamins et al. 2003; Day et al. 2002). אכן, בחינת רמת ההורמון, הראתה ירידה ברמת האוקסין החופשי בפקעים אלו לעומת פקעי שפע. ממצאים אלו מכוונים לכך שרמה גבוהה של ההורמון בפקעי שפע משחקת תפקיד בכך שהפקע אינו מקבל סיגנל לפריחה בהשפעת עומס רב של יבול. סילוק עודפי אוקסין משחק לפיכך תפקיד חשוב בהפיכת פקע שפע לפקע שפל. ממצאים אלו, והמסקנה מהם מתאימים לתיאוריית Auxin Auto-inhibition (ATA) שהוצעה ע"י F. Bangerth, והורחבה לאחרונה לתפקיד ההורמון בעיכוב פריחה (Bangerth 1989; 2006; Caaejas and Bangerth 1997; Smith and Samach 2013). אכן, דווח בעבר כי שימוש במעכבי טרנספורט פולארי של אוקסין עודד פריחה בעצי פרי (Bangerth 2006). בעבודה המתבצעת בימים אלו במעבדה נראה כי שינויים מכוונים ברמת סידן מביאים לשינויים מעניינים בביטוי גנים לפריחה.

ביטוי הגן *SPL5* בפקעי הדרים, ובעלים של תפוח הראה, כצפוי עליה באברי עץ שפל לעומת עץ שפע, בהתאם לתפקידו של הגן במעבר מפאזה יובנילית לפאזה בוגרת, ובעידוד פריחה עונתית (Fornara and Coupland 2009). בהתחשב בתפקידו כבקר-על, לא מפתיע כי הבדלים בין פקעי שנת שפע לפקעי שנת שפל ניכרים כבר בחודש מאי, הרבה לפני תקופת האינדוקציה לפריחה, ולפני שההבדלים בביטוי גנים המבוקרים על ידו ניכרים (Goldberg-Moeller et al. 2013; Munoz-Fambuena et al. 2011; Shalom et al. 2012). בעבודה זו הוכח כי *SPL5* מהדרים אכן פעיל בארבידופסיס בהקדמת פריחה, וכי הוא אכן מבוקר ע"י miR156. ההוכחה כי הוא פעיל גם בהדרים עדיין מתעכבת. אחת השאלות המעניינות נוגעת לאופן בקרתו. למרות שלא נצפה הבדל בביטוי miR156 בין פקעי שנת שפע לשפל, נראה כי לאורך העונה, ביטוי הגן רציפרוקאלי לביטוי ה-miR. בעבודה זו התגלו ממצאים מעניינים לגבי תעתיקים באזור הגן, במיוחד שחלק מהתעתיקים נבעו

מביטוי הגן PP2C המשחק תפקיד בהעברת הסיגנל ל-ABA. בהתחשב בממצאים לגבי השינויים בהורמון זה בפקעים לאחר דילול, היה מעניין לבחון את האפשרות כי יש בקרה הדדית בין שני הגנים. הממצאים על פניהם שוללים אפשרות זו, שכן ביטוי ביתר או anti-sensing של PP2C לא הביא לפנוטיפים המצופים. גם מוטנטים במערכת ה-NMD שתפקידה לסלק תעתיקים מוטעים והוצע לגביה תפקיד בקרתי בביטוי גנים לא הניבה תוצאות מעניינות.

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<u>טבלת סיכום תכנית מחקר 203-0870-12</u>. סירוגיות בהדרים: לימוד תהליכים המשתנים בין שנת שפע לשנת שפל

1. מטרות המחקר לתקופת הדו"ח תוך התייחסות לתכנית העבודה.

מטרות המחקר הנן:

1. ביצוע אנליזה גנומית משווה של פקעים מעץ בשנת שפע ממנו נקטף הפרי לעומת עץ ביקורת
2. אימות תוצאות של האנליזות הגנומית ברמה המולקולארית והביוכימית.
3. בחינת האפשרות כי הגן ההומולוגי ל-*SPL5* משחק תפקיד בבקרת הסירוגיות.

בתקופה המתוארת בדו"ח השגנו מטרות מס' 1, 2 3 באופן מלא.

2. עיקרי הניסויים והתוצאות שהושגו בתקופה אליה מתייחס הדו"ח.

1. ביצוע אנליזה גנומית וניתוח שלה
2. אימות תוצאות חשובות ברמה מולקולארית וביוכימית
3. אנליזת ביטוי ואנליזה פונקציונאלית של הגן *SPL* ובחינת אפשרויות שונות לבקרה שלו בהקשר לפריחה

3. המסקנות המדעיות וההשלכות לגבי יישום המחקר והמשכו.

הושגה הבנה לגבי מעורבות אפשרית של ההורמונים אוקסין ו-ABA בהגדרת מצב הפקע ומוכנותו לקבל את סיגנל הפריחה במועד האינדוקציה לפריחה. על סמך תוצאות שהושגו במסגרת התכנית, אנו מבצעים ניסויים פיזיולוגים שמטרתם להוכיח כי אכן שינויים ברמת ההורמונים עשויים לשנות מוכנות לפריחה ברמת הפקע. שימוש במעכבי טרנספורט פולארי הוצע בעבר. העבודה מעלה את האפשרות לשנות טרנספורט פולארי של אוקסין, וע"י כך מוכנות לפריחה, ע"י שינויים ברמת הסידן התוך תאי.

4. הבעיות שנותרו לפתרון ו/או השינויים שחלו במהלך העבודה (טכנולוגיים, שיווקיים ואחרים); התייחסות המשך המחקר לגביהן.

לאחר הוכחת התכנות מספר טיפולים במערכת מודל (ענפים ופקעים מנותקים, ענפים על עץ שלם), יש לנסות יישום טיפולים בעץ השלם וברמת המטע.

5. האם הוחל כבר בהפצת הידע שנוצר בתקופת הדו"ח - <u>יש לפרט</u>: פרסומים - כמקובל בביבליוגרפיה, פטנטים - יש לציין מס' פטנט, הרצאות וימי עיון - יש לפרט מקום ותאריך.

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מרבית מאמר זה מכיל את הממצאים מהתכנית הקודמת, מלבד הממצאים הנוגעים לביטוי *SPL* שהושגו במסגרת התכנית הנוכחית

הרצאה נובמבר 2011, The International Symposium on Genomics and Functional Genomics of Fruit Crops, Wuhan China

6. פרסום הדו"ח: אני ממליץ לפרסם את הדו"ח: (סמן אחת מהאופציות)

- רק בספריות
- <u>ללא הגבלה (בספריות ובאינטרנט)</u>
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RESEARCH PAPER

Fruit load induces changes in global gene expression and in abscisic acid (ABA) and indole acetic acid (IAA) homeostasis in citrus buds

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Abstract

Many fruit trees undergo cycles of heavy fruit load (ON-Crop) in one year, followed by low fruit load (OFF-Crop) the following year, a phenomenon known as alternate bearing (AB). The mechanism by which fruit load affects flowering induction during the following year (return bloom) is still unclear. Although not proven, it is commonly accepted that the fruit or an organ which senses fruit presence generates an inhibitory signal that moves into the bud and inhibits apical meristem transition. Indeed, fruit removal from ON-Crop trees (de-fruiting) induces return bloom. Identification of regulatory or metabolic processes modified in the bud in association with altered fruit load might shed light on the nature of the AB signalling process. The bud transcriptome of de-fruited citrus trees was compared with those of ON- and OFF-Crop trees. Fruit removal resulted in relatively rapid changes in global gene expression, including induction of photosynthetic genes and proteins. Altered regulatory mechanisms included abscisic acid (ABA) metabolism and auxin polar transport. Genes of ABA biosynthesis were induced; however, hormone analyses showed that the ABA level was reduced in OFF-Crop buds and in buds shortly following fruit removal. Additionally, genes associated with Ca^{2+}-dependent auxin polar transport were remarkably induced in buds of OFF-Crop and de-fruited trees. Hormone analyses showed that auxin levels were reduced in these buds as compared with ON-Crop buds. In view of the auxin transport autoinhibition theory, the possibility that auxin distribution plays a role in determining bud fate is discussed.

Key words: Abscisic acid, alternate bearing, auxin, bud, citrus, flowering, fruit load.

Introduction

Fruit trees exhibit two major multiannual reproductive strategies (Goldschmidt, 2013). In the first, the amount of fruit produced allows a sufficient amount of vegetative growth to support production of an ample number of flowers during the following year (return bloom). Such trees, including fig and some orange and grapefruit cultivars, are defined as regular bearers. They are characterized by a relatively stable multiannual yield, and usually possess efficient mechanism(s) to control excess fruit production. A second strategy is also used by trees that bear a heavy fruit load (ON-Crop) in one year, which inhibits return bloom and vegetative growth the next year (Monselise and Goldschmidt, 1982). Thus, the second year is characterized by low yield (OFF-Crop) and high vegetative growth. Such trees, including olive, pistachio, mandarins, and many others, are defined as alternate or biannual bearers and they are usually characterized by low self-thinning ability (Goldschmidt, 2013). Alternate bearer cultivars present a serious economic problem to fruit growers. Therefore, chemical or manual fruit thinning are common practices in their cultivation (Dennis, 2000). In citrus culture, low temperatures

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during the autumn and winter are a major factor in inducing flowering (Valiente and Albrigo, 2004; Knauer *et al.*, 2011). Optimal flowering density is achieved only upon accretion of sufficient cool hours. It is assumed that a heavy fruit load prevents recognition of the low-temperature flowering inductive signal and/or blocks later stages of inflorescence, such as bud break (Albrigo and Galán-Saúco, 2004; Verreynne and Lovatt, 2009). As expected, fruit load affects the expression of flowering control genes, *FT*, *LFY*, *AP1*, *TFL*, and *miR156*-regulated *SQUAMOSA PROMOTER BINDING* (*SPL5*) in leaves and buds of citrus (Muñoz-Fambuena *et al.*, 2011, 2012*b*; Shalom *et al.*, 2012) as well as in mango and apple (Kotoda *et al.*, 2010; Nakagawa *et al.*, 2012).

The mechanism by which heavy crop load affects return bloom is not fully understood. The developing fruit provides a strong sink for photoassimilates. It was therefore thought that depletion of photoassimilates, especially carbohydrates from the bud, prevents flowering induction, a hypothesis known as the nutritional theory (Goldschmidt *et al.*, 1985; Goldschmidt, 1999). Sucrose was shown to play a regulatory role in *Arabidopsis* flowering control (Eriksson *et al.*, 2006), but whether sugars indeed play a regulatory role in flowering induction under various fruit loads in fruit trees has been a controversial issue for many years (Hilgeman *et al.*, 1967; Jones *et al.*, 1970, 1974; Goldschmidt and Golomb, 1982; Li *et al.*, 2003*a*, *b*). Recent work has shown that trehalose metabolism and its product trehalose-6-phosphate were involved in flowering control in *Arabidopsis* (van Dijken *et al.*, 2004; Wahl *et al.*, 2013). It was also shown that two genes encoding enzymes associated with trehalose metabolism were induced in OFF-Crop buds (Shalom *et al.*, 2012). In addition to the nutritional control of alternate bearing (AB), it might well be that the fruit itself, or an organ which senses fruit presence, generates an inhibitory signal (AB signal) which moves into the bud and prevents flowering induction (Bower *et al.*, 1990; Tálon *et al.*, 1997). Fruit thinning or complete removal (de-fruiting) from ON-Crop trees induces return bloom (Monselise and Goldschmidt, 1981), thus providing support for this notion. Gibberellin (GA) is known to inhibit flowering in many perennials (Goldschmidt and Samach, 2004; Bangerth, 2009). However, while exogenous application of GA prevents flowering (Goldschmidt *et al.*, 1997; Muñoz-Fambuena *et al.*, 2012*a*; Goldberg-Moeller *et al.*, 2013), the question of whether GA acts endogenously to inhibit flowering is still open. The involvement of abscisic acid (ABA) in the regulation of return bloom is even less clear (Jones *et al.*, 1976; Goldschmidt, 1984; Koshita *et al.*, 1999; Okuda, 2000). Polar auxin transport from a dominant sink was also suggested as a possible mobile signal affecting flowering (Caaejas and Bangerth, 1997; Smith and Samach, 2013).

Fruit load might act at various developmental stages such as flowering induction, transition of the shoot apical meristem, and subsequent stages of flower development and bud break (Verreynne and Lovatt, 2009). Regardless of the source of the AB signal and its nature, it must be recognized by its receptor in the bud which in turn must make the 'decision' of whether to proceed to inflorescence or not. In order to investigate metabolic and regulatory processes taking place in the bud and affected by fruit load, the transcriptome of buds from ON- and OFF-Crop trees was recently compared during three developmental stages. Changes in metabolic and regulatory pathways, including photosynthesis, and in flavonoid and trehalose metabolism were identified (Shalom *et al.*, 2012). However, this work was biased due to the use of an Affymetrix Citrus Gene-Chip array that contained ~15 500 genes. In fact, with the exception of trehalose metabolism, no other regulatory pathways were identified. In the current work, a complementary approach was taken by comparing the transcriptome of buds of de-fruited trees with those of ON-Crop trees. The genomic analysis was non-biased, as it was based on RNA-deep sequencing. It was possible to identify an increase in ABA-metabolizing genes, accompanied by a decrease in ABA levels and those of its catabolites in buds of de-fruited trees. Moreover, a remarkable increase in the expression of genes encoding proteins associated with calcium-dependent auxin polar transport and a reduction in bud endogenous auxin levels following de-fruiting were identified. The results are discussed in light of the previously suggested auxin transport autoinhibition (ATA) theory (Bangerth, 1989) and its role in AB (Smith and Samach, 2013).

Materials and methods

Plant material and sample collection

Plant material was collected from a commercial orchard of 15-year-old Murcott mandarin (*Citrus reticulate* Blanco) trees grafted on sour orange (*Citrus aurantium* L.), located in the central coastal area of Israel, during the years 2011 (an ON year) and 2012 (an OFF year). Although most of the trees in the orchard bore similar yields in a given year, some were aberrant and showed an opposite AB trend. These and nearby trees with the opposite yield status were selected. Overall, nine triplets of trees were chosen, with each triplet (two ON trees and a nearby OFF tree) being considered one biological replicate. Fruits were completely removed (de-fruiting) on 22 August 2011 from one of the ON trees in each triplet, and this tree was labelled DEF. Samples were collected 1 d prior to de-fruiting (Time 0, from ON and OFF trees), 1 week following de-fruiting (Time 1, from ON and DEF trees), 2 weeks following de-fruiting (Time 2, from ON and DEF trees), and 4 weeks following de-fruiting (Time 4, from ON, OFF, and DEF trees).

The three most extreme conditions of spring flush flowering were compared (see fig. S2 in Shalom *et al.*, 2012): fruit-bearing flush of an ON tree, fruitless flush of an OFF tree, and de-fruited flush of a DEF tree. Branches of each of these conditions were collected from the southeast side of the trees, taken to the laboratory on ice, and buds were separated and immediately frozen in liquid nitrogen. The percentages of generative, mixed, and vegetative shoots were determined for all branches splitting off from one major 50–60 mm diameter branch located on the southeast side of the tree, during peak blossoming time (usually the first 10 d of April of the consecutive year). Selection of the branches to be sampled was done prior to bud break.

RNA extraction, quantification, and qPCR analyses

Total RNA was extracted, treated, and analysed from ~0.2 g of frozen bud tissue, and cDNA was synthesized, as previously described (Shalom *et al.*, 2012). Primers for the genes *CiFT2*, *CsLFY*, *SPL5*, *RbcS*, *LHCB3*, *PRK*, *PSB28*, *PSAD*, *SHM*, *Fd*, *NCED3*, *CAX*, *PBP1-like1*, *PBP1-like2*, *NPH3*, *CA-binding EF hand1*, *β-ACTIN*, and a dual-labelled probe for *CiFT2* were designed based on genomic

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and expressed sequence tag (EST) sequences (Phytozome, http://www.phytozome.net/; HarvEST, http://harvest.ucr.edu/) using Primer 3 software (Supplementary Table S1 available at *JXB* online). Real-time PCR was carried out as described (Shalom *et al.*, 2012). For the *CiFT2* dual-labelled probe reaction, real-time PCR was carried out as described by Shalom *et al.* (2012). The mRNA levels of trehalose biosynthetic genes and flavonoid biosynthetic genes (Supplementary Table S2) were determined by nCounter analysis (Nanostring Technologies, Seattle, WA, USA) at the VIB MicroArrays Facility (Leuven, Belgium), as described by Shalom *et al.* (2012).

RNA deep sequencing

Extracted RNA integrity was determined by Agilent Bioanalyzer (Santa Clara, CA, USA) according to the manufacturer's instructions. A 2 µg aliquot of total RNA from each sample was prepared and used for cDNA library constructions using the TruSeq mRNA sample preparation kit according to the manufacturer's protocol (Illumina Inc., San Diego, CA, USA, REF 15025062). The libraries (10 pmol) were run on a single read 100 nucleotide run on the HiSeq 2000 (Illumina Inc.) on six lanes. Raw fastq files were quality checked using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc), and adaptor sequences were removed using fastq-mcf (https://code.google.com/p/ea-utils/wiki/FastqMcf). They were then aligned to the orange (*Citrus sinensis*) genome database (Xu *et al.*, 2013; http://citrus.hzau.edu.cn/orange/) using TopHat2 (Kim *et al.*, 2013). FPKM (fragments per kilobase of transcript per million mapped reads) values, which normalize the read count by the length of the fragment and the total number of mapped reads, were calculated, and differential expression was checked using Cufflinks (Trapnell *et al.*, 2010). A hierarchical clustering heatmap and a 2D principle component analysis (2D PCA) plot were generated by MATLAB (Mathworks, Cambridge, UK), using the log-FPKM values of each gene. For gene ontology (GO) analysis, sequences were blasted against the UniRef90 database (Suzek *et al.*, 2007), by which a number of GO annotations were derived for each gene. Singular enrichment analysis (SEA), which lists enriched GO terms, was performed [false discovery rate (FDR) ≤0.05] for the five best GO terms of each gene using the AgriGo interface (http://bioinfo.cau.edu.cn/agriGO/index.php). Differentially expressed genes were also functionally annotated via the automated Mercator pipeline (Lohse *et al.*, 2013) (http://www.gabipd.org/biotools/mercator/) and displayed on diagrams of metabolic and other processes using MapMan (Usadel *et al.*, 2009; http://www.gabipd.org/projects/MapMan/).

Hormonal content analysis

About 50 mg of bud tissue from each tested sample were lyophilized and homogenized to a fine powder in liquid nitrogen using a mortar and pestle. Quantification of ABA, ABA metabolites, and indole acetic acid (IAA) was conducted at the National Research Council of Canada (Saskatoon, Saskatchewan, Canada) according to published protocols (http://www.nrc-cnrc.gc.ca).

Protein extraction and western blot analysis

Proteins were extracted from buds, quantified, and analysed by western blot analysis using specific primary antibodies, raised against the following proteins: Rubisco complex large (RbcL) and small (RbcS) subunits, ferredoxin (Fd), chlorophyll *a*/*b* protein [light-harvesting chlorophyll *a*/*b* complex II (LHCII)], and D1 protein (PsbA), as described by Maayan *et al.* (2008).

Statistical analysis

The statistical analyses used for qPCR results, hormone analyses, and inflorescences numbers were one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparison tests as implemented in the software JMP version 10 (SAS Institute).

Results

Fruit removal (de-fruiting) induces back flowering

Normally, fruit load status is similar among most trees of the orchard—trees bear either a heavy crop (ON-Crop year) or a low crop (OFF-Crop year). A few trees, however, show the opposite trend, allowing the collection of samples from nearby trees bearing either high fruit load or low fruit load. In order to detect changes which might play a role in converting ON- to OFF-Crop buds, fruits were completely removed from ON-Crop trees in August and buds were collected 1, 2, and 4 weeks following de-fruiting. The effect of the treatment was verified the following spring by counting the number of inflorescences and vegetative shoots (Fig. 1). Citrus trees bear two major types of inflorescences: generative (leafless) and mixed (leafy; flowers and leaves in various ratios). ON-Crop trees had significantly less generative inflorescences compared with OFF-Crop trees and DEF trees (22% versus 70–75%) and more vegetative shoots (43% versus 3–4%). As expected, no significant differences were detected in mixed-type inflorescences. Fruit counting during harvest time showed that ON-Crop trees yielded 1635 ± 98 fruits per tree while OFF-Crop trees yielded 36 ± 12 fruits per tree.

Fruit removal alters expression of flowering control and other genes in the bud

In order to determine how quickly buds responded to de-fruiting, the mRNAs levels of several flowering control genes were quantified during the course of the experiment (Fig. 2). The *Citrus* genome contains three *FT* genes, but only the expression of *CiFT2* correlated well with tree flowering intensity (Nishikawa *et al.*, 2007; Shalom *et al.*, 2012). In buds of ON-Crop trees, *CiFT2* mRNA levels were relatively low, and remained unchanged. In contrast, *CiFT2* mRNA was ~15-fold higher in buds of OFF-Crop trees. One week following de-fruiting, the expression of the gene in the buds of the DEF

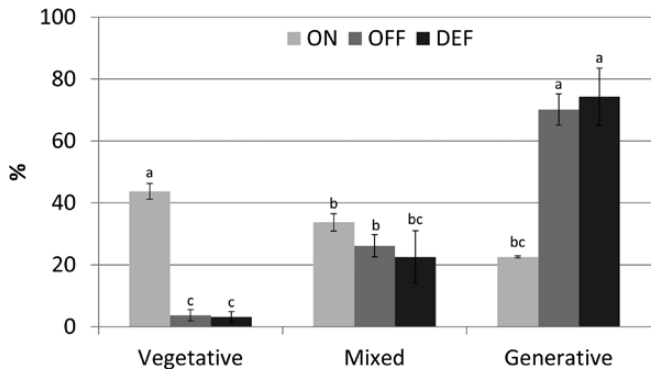

Fig. 1. Fruit load affects flowering intensity the following year. Vegetative shoots, generative inflorescences containing only flower buds, and mixed inflorescences containing flower buds and leaves in various ratios were counted during flowering peak in trees which carried a heavy yield (ON) or light yield (OFF), and in de-fruited trees (DEF). The numbers are mean values of three independent biological replicates ±SE. Different letters represent a significant difference ($P \leq 0.05$).

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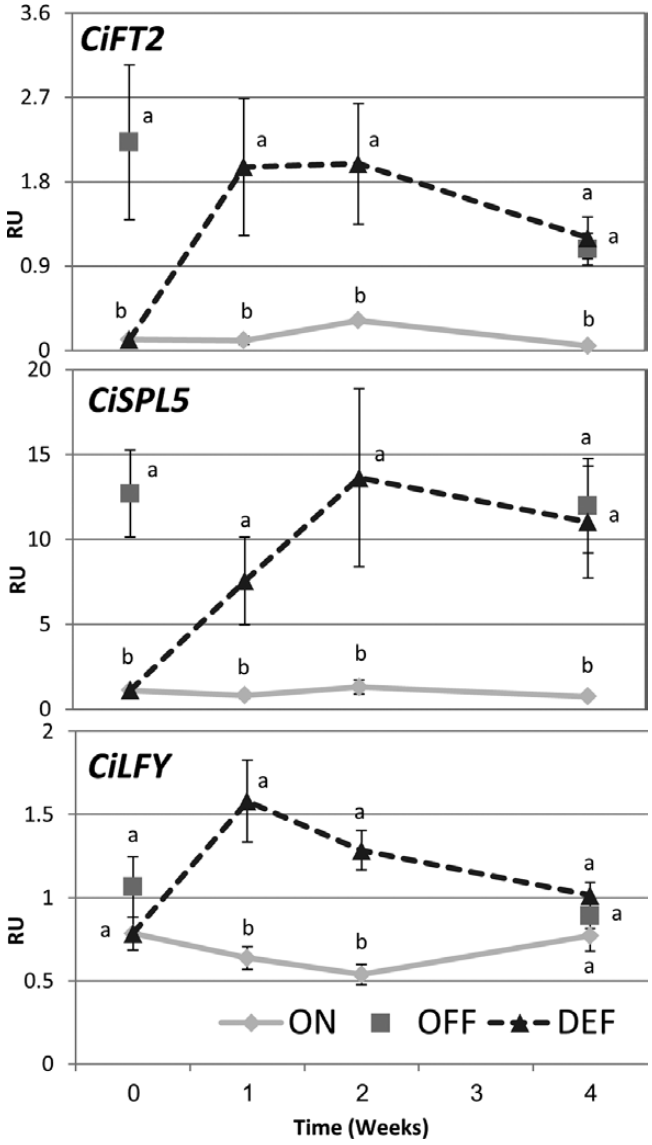

Fig. 2. Fruit removal alters the expression of flowering control genes in buds. The mRNA levels (RU, relative units) of the indicated genes were determined in ON-Crop (ON), OFF-Crop (OFF), and de-fruited (DEF) trees at the indicated weeks following de-fruiting. The numbers are mean values of three independent biological replicates ±SE. Different letters represent a significant difference ($P \leq 0.05$) between the states at the same time point.

trees was similar to that of OFF-Crop buds, and it remained at this level during the entire test period. The mRNA levels of *LFY* were similar in buds of ON- and OFF-Crop trees, but it increased 3-fold within 1 week in DEF buds and returned to its basal level after 4 weeks. It was previously shown that *miR156*-regulated *SPL5* displayed elevated mRNA levels in OFF-Crop buds; thus, it may act as a positive inducer of flowering in *Citrus* trees (Shalom *et al.*, 2012). As expected, *SPL5* mRNA levels were ~14-fold higher in buds of OFF-Crop trees as compared with ON-Crop buds. DEF buds displayed increased mRNA as compared with OFF-Crop buds within 2 weeks of de-fruiting. The expression of genes associated with trehalose and flavonoid metabolism was elevated in ON- and OFF-Crop buds (Shalom *et al.*, 2012). As expected, the mRNA levels of trehalose phosphate synthase and trehalose phosphate phosphatase were reduced by 2- and 4-fold, respectively following de-fruiting (Supplementary Fig. S1 at *JXB* online). The mRNA levels of flavonoid biosynthesis genes, *UF3GT*, *4CL*, *CHS*, and *CHI*, were induced by the treatment. De-fruiting resulted in a reduction of *C4H* mRNA levels in the ON-Crop trees.

Fruit removal induces rapid changes in the bud global gene expression

The results indicated that the transition of the bud from an ON to an OFF state took place relatively quickly. In order to analyse the metabolic and regulatory pathways playing a role in this transition, changes in global gene expression were analysed in buds before de-fruiting (Time 0) and 1 (Time 1), 2 (Time 2), and 4 (Time 4) weeks after de-fruiting (Fig. 3A). In addition, buds of OFF-Crop trees were also analysed at Time 0 and Time 4. The minimum number of reads per sample was ~15 million and the maximum was ~40 million, indicating a deep and satisfactory coverage of the existing transcripts (Supplementary Table S3 at *JXB* online). Overall, the number of transcripts in all libraries was ~14 400. Hierarchical cluster analysis (Fig. 3B, left) and 2D PCA (Fig. 3B, right) were performed based on the log-FPKM values of each sample. Results of both analyses showed that during all time points, including Time 1, the transcript profiles of DEF buds were more closely related to those of OFF-Crop buds than to those of ON-Crop buds, thus supporting the notion that the transition from an ON bud to an OFF bud was relatively quick following de-fruiting. In order to analyse the metabolic and regulatory pathways mediating the ON bud to OFF bud transition, two major comparisons were made (the ratios for all possible comparisons in the experiment are presented in Supplementary File 2 at *JXB* online). First, Time 4 included the three fruit load states, ON, OFF, and DEF. Therefore, the genes that were up- or down-regulated ($P \leq 0.05$) in OFF or DEF buds in comparison with ON-Crop buds were identified (Fig. 4A; Supplementary File 2). Overall, 997 genes in OFF-Crop buds and 797 genes in DEF buds were down-regulated relative to ON-Crop buds at Time 4, with 615 genes common to the two groups (OFF and DEF). Overall, 959 genes in OFF-Crop buds and 920 genes in DEF buds were up-regulated relative to ON-Crop buds at Time 4, with 564 genes common to the two groups (OFF and DEF) (Fig. 4A; Supplementary File 2). The second comparison aimed at identifying genes which showed alternation in their expression during the course of the experiment and also common or different pathways altered developmentally. For that, genes were clustered according to their expression patterns relative to Time 0. For ON-Crop and DEF buds, four clusters were identified (Fig. 4B; Supplementary File 2). For OFF-Crop buds, only two time points were analysed, and altered genes were either reduced or repressed (Supplementary File 2)

In order to identify common and unique metabolic and regulatory pathways in DEF and OFF-Crop buds which were altered in comparison with ON-Crop buds at Time 4, up-regulated genes (564+395 and 564+356, Fig. 4A) and

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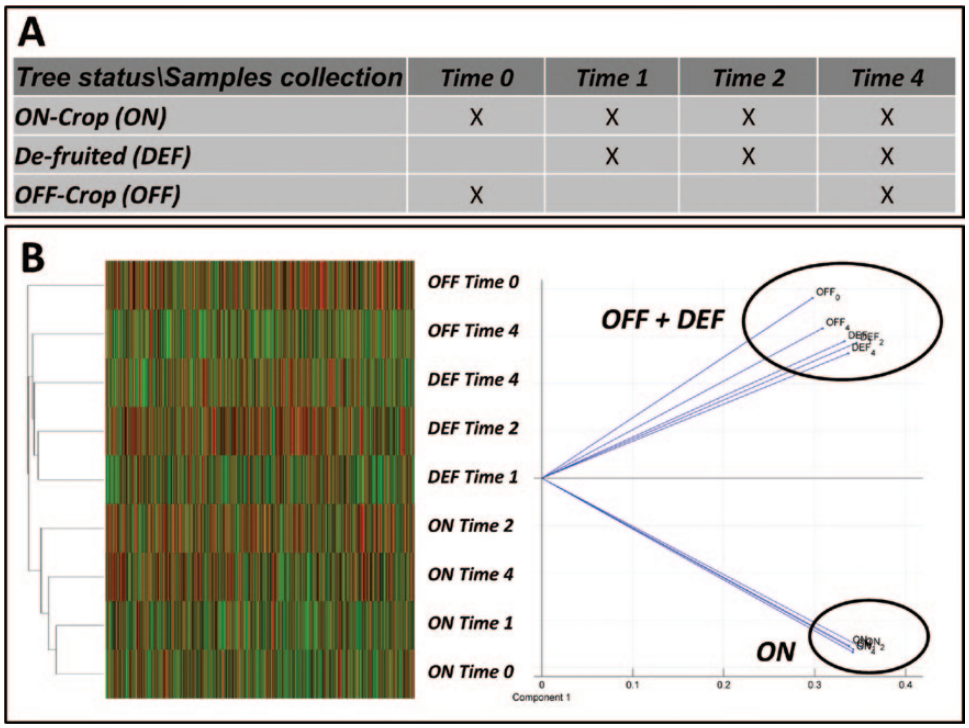

Fig. 3. Global gene expression in buds of de-fruited (DEF) trees is similar to that of buds of OFF-Crop (OFF) trees. The experimental design and sample collection (A). Hierarchical clustering heatmap (left panel) and 2D principle component analysis (right panel) plots were generated using the log-*FPKM* value of each gene for all samples, as indicated in A (B). (This figure is available in colour at *JXB* online.)

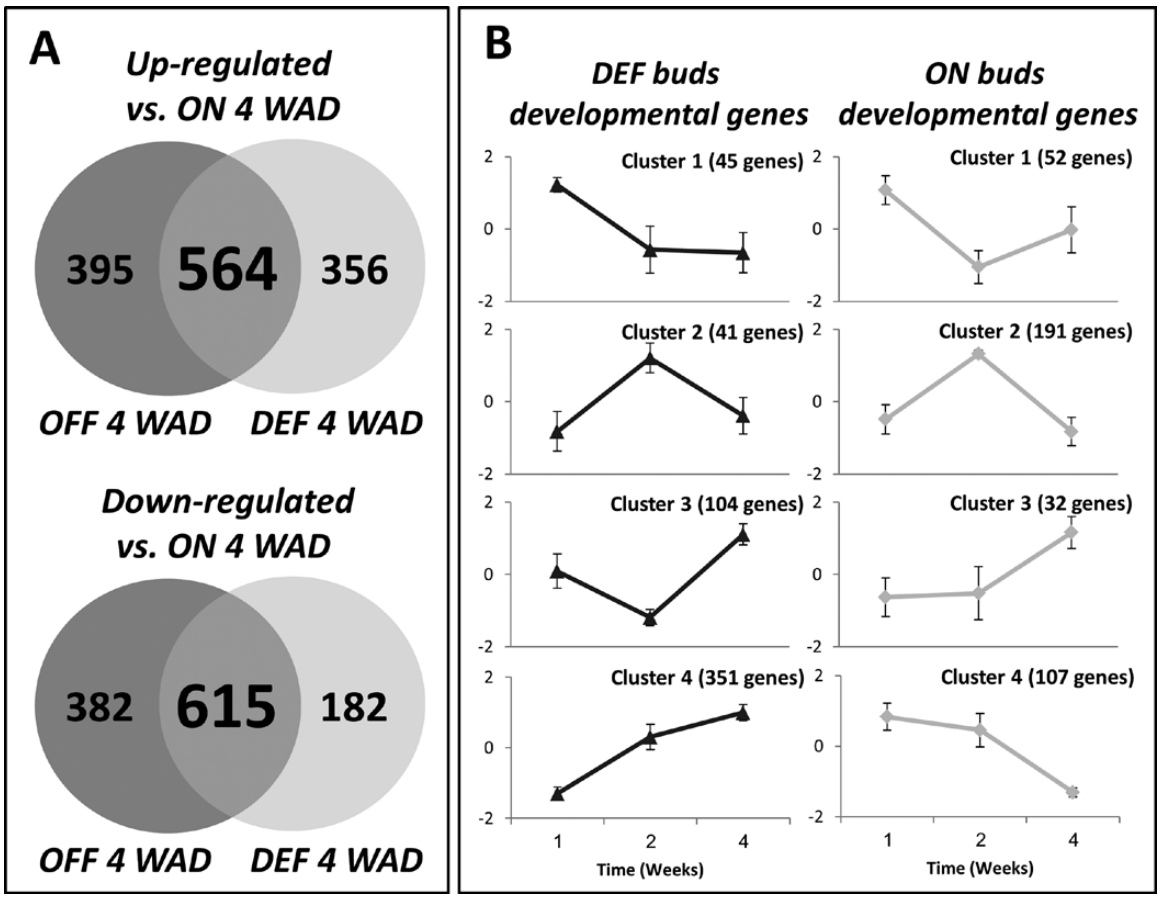

Fig. 4. Venn diagrams of differentially expressed genes and clustering analysis of developmentally altered genes. The number of genes in buds of OFF-Crop (OFF) and de-fruited (DEF) trees which were significantly ($P \leq 0.05$) up-regulated (left) or down-regulated (right) compared with ON-Crop buds at Time 4 (A). Clustering analysis of genes which were significantly ($P \leq 0.05$) altered developmentally during the course of the experiment in comparison with Time 0 as generated by Expender (http://acgt.cs.tau.ac.il/expander/overview.html) using the Click Algorithm (B). All genes are listed in Supplementary File 2 at *JXB* online.

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down-regulated genes (615+382 and 615+182, Fig. 4A) were GO annotated and further analysed using SEA (AgriGO). Table 1 presents these common processes. However, in a few cases, unique GO terms were included under the same general process either because of redundancy or because they were part of one process. The analysis indicated that the great majority of common processes which were enriched in DEF/OFF-Crop buds were related to light sensing (such as response to light stimulus/intensity and response to far red/blue and red light), photosynthesis (such as dark and light reactions and photosynthetic electron transport chain), chloroplast reorganization (such as plastid localization, chloroplast relocation and organization), response to carbohydrate stimulus (i.e. sucrose and disaccharide), ion homeostasis (such as cation homeostasis and transport, proton transport), and the pentose-phosphate cycle. Fewer genes were down-regulated in DEF/OFF-Crop buds and belonged to several secondary metabolic pathways such as the terpenoid and phenylpropanoid metabolic process, and oxidative reduction. Major unique processes induced in OFF-Crop buds included starch biosynthesis, carbohydrate metabolism, glycoside and glycosinolate metabolism, and water homeostasis (Supplementary Table S4 at *JXB* online). Considerably more unique processes were induced in DEF buds as compared with OFF-Crop buds (Supplementary Table S4). They included regulation of peptidase activity, regulation of dephosphorylation, and salicylic acid metabolism. Down-regulated unique processes in DEF buds included amino acid/amine metabolism and aromatic compound metabolism (Supplementary Table S4). Responses to biotic stress were either induced or repressed in DEF buds. Unique genes down-regulated in OFF-Crop buds could not be GO annotated, due to their low number.

GO annotation and SEA were also performed to developmentally altered genes compared with Time 0 in ON-Crop, OFF-Crop, and DEF buds (Fig. 4B). Significantly altered biological processes (FDR ≤0.05) could be identified for genes of clusters 3 and 4 (DEF buds), cluster 2 (ON-Crop buds), and up/down regulated genes of OFF-Crop buds. No significant biological processes could be identified even under FDR ≤0.1 in genes of the other clusters. Cluster 4 of DEF buds included processes involved in light responses and photosynthesis (Supplementary Table S5 at *JXB* online). A considerable induction was also detected in genes of respiratory burst and in those involved in responses to abiotic stresses. Genes up-regulated in OFF-Crop buds from Time 0 to Time 4 were annotated into light responses, photosynthesis, and response to carbohydrate stimulus (Supplementary Table S6). Down-regulated genes in OFF-Crop buds from Time 0 to Time 4 included responses to jasmonic acid (JA) and ethylene (Supplementary Table S7). Other processes included amino acid and amine metabolism, and responses to abiotic stresses. Cluster 3 of DEF buds included 104 genes that could be annotated into different biological processes, a few of them related to development and morphogenesis, such as trichoblast and root differentiation, floral organ development, and shoot morphogenesis (Supplementary Table S8). Cluster 2 of ON-Crop buds was enriched in genes associated with lipid transport (Supplementary Table S9).

An additional analysis for developmentally regulated genes was aimed at identifying common genes in clusters showing a similar expression pattern in OFF-Crop and DEF buds and opposite patterns in ON-Crop buds. The first comparison included cluster 4 of DEF buds, up-regulated genes of OFF-Crop buds, and cluster 4 of ON-Crop buds (Supplementary Fig. S2A at *JXB* online). However, only one unknown gene was common among the three states. The second comparison included cluster 1 of DEF buds, down-regulated genes of OFF-Crop buds, and cluster 3 of ON-Crop buds (Supplementary Fig. S2B). Only the expression of three genes was common; two of them were homologous to terpene (nerolidol) syntheses (*Cs2g07240* and *Cs2g07250*).

Photosynthetic genes and proteins are up-regulated in response to fruit removal

Consistent with a previous report (Shalom *et al.*, 2012), the above-described results and MapMan analysis of differentially expressed genes in DEF versus ON-Crop buds at Time 4 showed that genes associated with light reactions, the Calvin–Benson cycle, and to a lesser extent photorespiration were induced (Supplementary Fig. S3 at *JXB* online). Fold changes of 41 photosynthetic genes in DEF and OFF-Crop buds relative to ON-Crop buds are shown in Fig. 5A. Out of 41 differentially expressed genes, 38 were up-regulated while only three were down-regulated at at least one time point throughout the experiment. Validation of the above results by qPCR analyses was performed for seven genes, *Ribulose bisphosphate carboxylase-small subunit* (*RbcS*), *Light-harvesting chlorophyll B-binding protein* (*LHCB3*), *Phosphoribulokinase* (*PRK*), *Photosystem II reaction centre PSB28* (*PSB28*), *Photosystem I subunit D* (*PSAD*), *Serine hydroxymethyltransferase* (*SHM*), and *Ferredoxin* (*Fd*) (Fig. 5B). The mRNA levels of all these genes were significantly higher in buds of OFF trees as compared with ON trees at Time 4. As expected, gene expression increased in DEF buds by 2.6- to 5.2-fold within 1 week of fruit removal. Furthermore, western blot analyses using specific antibodies raised against RbcS, Fd, LHCII, and PsbA showed that their protein level was higher in OFF-Crop buds relative to ON-Crop buds, and increased in DEF buds (at Time 4) following fruit removal (Fig. 5C). However, RbcL protein levels remained unchanged in DEF and OFF-Crop buds relative to ON-Crop buds.

Fruit removal-induced changes in ABA-metabolizing genes

Results of genomic analysis showed that genes homologous to *9-cis-epoxycarotenoid dioxygenase* (*NCED*), coding for this rate-limiting enzyme of ABA biosynthesis (Supplementary Fig. S5 at *JXB* online), were higher in buds of OFF-Crop buds relative to ON-Crop buds (Fig. 6A). In *Arabidopsis*, the *NCED* gene family comprises nine members, and the roles of *NCED2*, *3*, *5*, and *6* in ABA biosynthesis were demonstrated (Tan *et al.*, 2003). The *Citrus* genome contained nine highly homologous genes, and changes in three of them were detected in genomic analysis: *Cs5g14370.1*, homologous to *NCED3*, *Cs5g14370.1*, homologous to *NCED1*, and

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Table 1. *Gene ontology categorization of common genes up- or down-regulated in buds of OFF-Crop and de-fruited (DEF) trees relative to buds of ON-Crop trees at Time 4*

General process	GO term	Description	Ref item	OFF				DEF			
				Query item	*P*-value	FDR	Fold enrichment[a]	Query item	*P*-value	FDR	Fold enrichment[a]
Up-regulated											
Response to light	GO:0010218	Response to far red light	25	11	2.80E-07	0.00057	6.48	14	7.20E-11	9.40E-08	8.51
	GO:0010114	Response to red light	47	14	1.90E-06	0.00089	4.39	17	3.80E-09	3.00E-06	5.50
	GO:0009639	Response to red or far red light	r	22	0.00023	0.024	2.31	22	0.00015	0.014	2.39
	GO:0009416	Response to light stimulus	380	44	0.00055	0.047	1.70	59	2.20E-09	2.20E-06	2.36
	GO:0009637	Response to blue light	27	7	8.20E-05	0.049	6.39	11	5.20E-07	0.00015	6.19
	GO:0009644	Response to high light intensity	70					20	1.80E-08	8.90E-06	4.34
	GO:0009642	Response to light intensity	88					23	1.10E-08	7.00E-06	3.97
	GO:0009314	Response to radiation	404					59	2.10E-08	9.30E-06	2.22
Photosynthesis and carbon fixation	GO:0015977	Carbon fixation	7	6	6.60E-07	0.00068	12.62	5	2.40E-05	0.0031	10.85
	GO:0009773	Photosynthetic electron transport in photosystem I	12	7	4.00E-06	0.0013	8.59	5	0.00067	0.038	6.33
	GO:0015979	Photosynthesis	128	33	2.90E-11	1.20E-07	3.80	47	9.80E-23	3.90E-19	5.58
	GO:0009767	Photosynthetic electron transport chain	31	10	2.60E-05	0.0059	4.75	11	2.60E-06	0.00055	5.39
	GO:0019684	Photosynthesis, light reaction	85	19	3.90E-06	0.0013	3.29	31	1.60E-15	3.10E-12	5.54
	GO:0019685	Photosynthesis, dark reaction	5	5	1.50E-06	0.00087	14.72				
	GO:0010207	Photosystem II assembly	36					13	2.60E-07	8.50E-05	5.49
	GO:0009765	Photosynthesis, light harvesting	13					8	3.50E-07	0.00011	9.35
Chloroplast	GO:0051667	Establishment of plastid localization	28	9	6.70E-05	0.011	4.73	8	0.00034	0.023	4.34
	GO:0051644	Plastid localization	28	9	6.70E-05	0.011	4.73	8	0.00034	0.023	4.34
	GO:0009902	Chloroplast relocation	28	9	6.70E-05	0.011	4.73	8	0.00034	0.023	4.34
	GO:0051656	Establishment of organelle localization	31	9	0.00016	0.02	4.27	8	0.00073	0.04	3.92
	GO:0009658	Chloroplast organization	81	18	7.60E-06	0.002	3.27	14	0.00083	0.044	2.63
	GO:0009657	Plastid organization	123	21	9.50E-05	0.014	2.51	20	0.00018	0.015	2.47
Response to carbohydrate stimulus	GO:0009744	Response to sucrose stimulus	47	12	5.60E-05	0.011	3.76	14	1.30E-06	0.00033	4.53
	GO:0034285	Response to disaccharide stimulus	48	12	7.10E-05	0.011	3.68	14	1.80E-06	0.00039	4.43
	GO:0009743	Response to carbohydrate stimulus	178	31	1.70E-06	0.00088	2.56	33	9.30E-08	3.30E-05	2.82
Cellular homeostasis	GO:0071704	Organic substance metabolic process	7	6	6.60E-07	0.00068	12.62	5	2.40E-05	0.0031	10.85
	GO:0019725	Cellular homeostasis	129	23	2.30E-05	0.0055	2.63	21	0.00012	0.012	2.47
	GO:0055082	Cellular chemical homeostasis	57	12	2.90E-06	0.0037	5.19	16	5.90E-07	0.00016	4.26
Ion homeostasis	GO:0006873	Cellular ion homeostasis	52	12	0.00016	0.02	3.40	14	5.00E-06	0.00095	4.09
	GO:0030003	Cellular cation homeostasis	50	11	0.00046	0.043	3.24	13	1.60E-05	0.0023	3.95
	GO:0055082	Cellular chemical homeostasis	57	15	4.70E-06	0.0014	3.87	41	0.00013	0.012	1.87
	GO:0006812	Cation transport	333	43	6.00E-05	0.011	1.90	14	0.00037	0.023	2.84
	GO:0006811	Ion transport	470	55	0.0001	0.014	1.72	52	0.00028	0.021	1.68
	GO:0010155	Regulation of proton transport	11	6	3.40E-05	0.0075	8.03	7	1.40E-06	0.00033	9.67
	GO:0055080	Cation homeostasis	64					13	0.00025	0.019	3.09
Pentose cycle	GO:0019253	Reductive pentose-phosphate cycle	5	5	1.50E-06	0.00087	14.72				
	GO:0006098	Pentose-phosphate shunt	65					13	0.0003	0.022	3.04
Response to biotic stress	GO:0010200	Response to chitin	90	16	0.00038	0.038	2.62	17	8.10E-05	0.0089	2.87
	GO:0050832	Defence response to fungus	123	20	0.00027	0.028	2.39	20	0.00018	0.015	2.47
Abiotic stress	GO:0009611	Response to wounding	128	20	0.00046	0.043	2.30	20	0.00031	0.022	2.37

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Table 1. *Continued*

General process	GO term	Description	Ref item	OFF				DEF			
				Query item	*P*-value	FDR	Fold enrichment[a]	Query item	*P*-value	FDR	Fold enrichment[a]
Down-regulated											
Secondary metabolism	GO:0019748	Secondary metabolic process	467	58	5.80E-05	0.042	1.73	55	1.20E-06	0.0011	2.04
	GO:0006721	Terpenoid metabolic process	118	23	1.20E-05	0.016	2.72	18	0.00017	0.028	2.64
	GO:0006720	Isoprenoid metabolic process	186	29	8.10E-05	0.049	2.17	8	0.00031	0.044	4.46
	GO:0009699	Phenylpropanoid biosynthetic process	143					23	1.00E-05	0.0035	2.78
	GO:0009698	Phenylpropanoid metabolic process	184					27	1.10E-05	0.0035	2.54
Various	GO:0055114	Oxidation reduction	1344	166	2.00E-10	7.40E-07	1.72	137	2.60E-09	7.50E-06	1.76
	GO:0042221	Response to chemical stimulus	1345	142	1.30E-05	0.016	1.47	120	1.00E-05	0.0035	1.54
	GO:0010035	Response to inorganic substance	462	58	4.30E-05	0.039	1.75				

[a] Fold enrichment as calculated based on GO-annotated genes (777, 753, 821, 662 in OFF-Crop up-regulated, DEF up-regulated, OFF-Crop down-regulated, DEF down-regulated, respectively) in the list of genes (959, 920, 997, 797 in OFF-Crop up-regulated, DEF up-regulated, OFF-Crop down-regulated, DEF down-regulated, respectively) per GO-annotated genes (11445) in the reference list (14400 genes).

Cs7g14820.1, homologous to *NCED4*. Among these three genes, *NCED3-like* induction was increased by ~4-fold in OFF-Crop buds relative to ON-Crop buds at Time 0, and was ~2-fold higher at Time 4. Fruit removal induced a 3-fold increase at Time 1 and its mRNA level remained higher relative to ON-Crop buds throughout the experiment. Induction of *NCED1-like* was seen only in OFF-Crop buds at Time 4, while *NCED4-like* was induced in DEF buds at Time 1 and 4 and in OFF-Crop buds at Time 4. Among the three genes, NCED3 is considered the major enzyme catalysing the rate-limiting step in ABA biosynthesis, and the mRNA levels of its *Citrus* counterpart were higher in OFF and DEF buds throughout the experiment. qPCR validation showed that *Cs5g14370.1* mRNA levels in OFF-Crop buds were significantly higher relative to ON-Crop buds, by ~4.5-fold at Time 0 and by 2.1-fold at Time 4 (Fig. 6B). The mRNA levels in DEF buds increased relative to ON-Crop buds by 3.2-fold at Time 1 and remained higher throughout the experiment. The transcriptomic data showed that the expression of a gene homologous to *PYR1*, a component of the ABA receptor, was reduced (Supplementary Fig. S6).

The levels of ABA and its catabolites were analysed at three time points following de-fruiting (Fig. 7). In addition to ABA and its isomer, *trans*-ABA (t-ABA), four catabolites were detected in the buds, 7'OH-ABA, ABA glucose ester (ABAGE), phaseic acid (PA), and dihydrophaseic acid (DPA), with t-ABA, PA, and ABAGE showing relatively high levels. ABA levels in ON-Crop buds were significantly higher relative to OFF-Crop buds, by 2.4-fold at Time 0 and by 3.3-fold at Time 4. In DEF buds, ABA levels decreased relative to ON-Crop buds by 1.3-fold at Time 1 and by 1.8-fold at Time 4. In addition, the levels of PA and ABAGE were significantly higher in ON-Crop buds relative to OFF-Crop buds by 4.5-fold and 4.6-fold at Time 1 and by 7.7-fold and 2.9-fold at Time 4, respectively. In DEF buds, PA and ABAGE levels decreased by 3.4-fold and 1.8-fold at Time 1 and by 4.1-fold and 3.5-fold at Time 4, respectively. The levels of DPA and t-ABA were generally higher in ON-Crop buds relative to OFF and DEF buds, especially at Time 4, but the differences were not significant.

Induction of calcium-related genes associated with auxin transport in DEF and OFF-Crop buds

Among the genes which showed relatively high levels of expression in OFF-Crop and DEF buds relative to ON-Crop buds were calcium-related genes (Fig. 8A; Supplementary File 2 at *JXB* online). The expression of most of these genes was induced 3- to 40-fold at all time points of the experiment. Many of these genes encode proteins containing an EF-hand domain. PINOID (PID)-binding protein (PBPs) are a subgroup of EF-hand proteins. PBP1 has been shown to play a role in auxin polar transport in response to changes in calcium levels (Benjamins *et al.*, 2003). Four citrus *PBP1-like* genes (out of the five found in the citrus genome) were induced in DEF and OFF-Crop buds relative to ON-Crop buds at all time points tested (Fig. 8A). Phylogenic analysis of these genes showed remarkable homology with *PBP1* and its closely related gene in *Arabidopsis* (Supplementary Fig. S8A).

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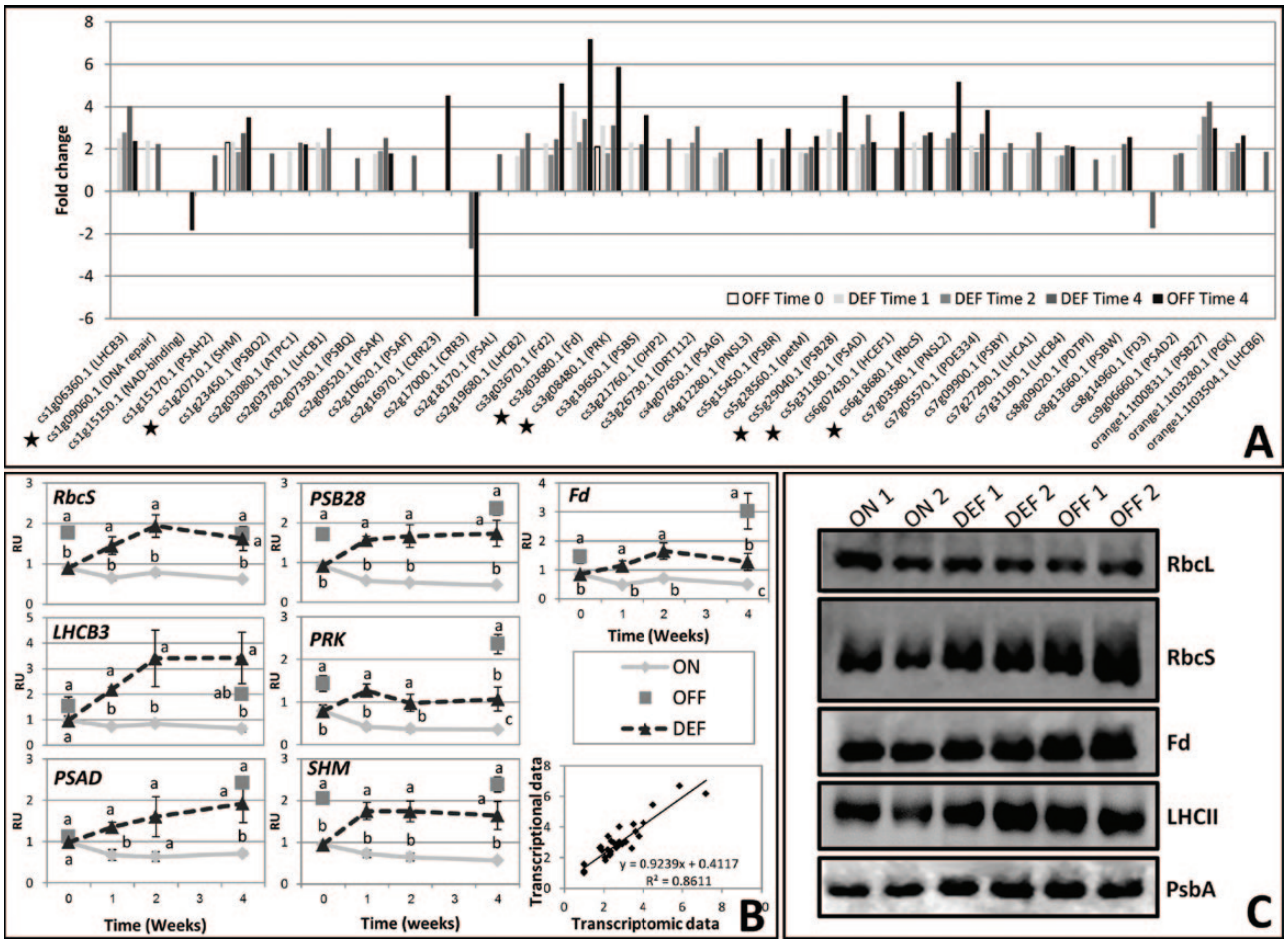

Fig. 5. The photosynthetic machinery is up-regulated in buds of OFF-Crop (OFF) and de-fruited (DEF) trees. Fold-change ($P \leq 0.05$) in the expression of photosynthetic genes (determined by MapMan analysis, see Supplementary Fig. S2 at *JXB* online) in buds of OFF and DEF trees relative to buds of ON-Crop (ON) trees at the indicated time points. Asterisks mark genes selected for validation by qPCR analyses, and specific genes are listed in Supplementary File 2 (A). Expression analysis of selected genes at the indicated weeks following de-fruiting, as determined by qPCR analyses (RU, relative units). The numbers are mean values of three independent biological replicates ±SE. Different letters represent a significant difference ($P \leq 0.05$) between the states at the same time point. The lower right graph shows the linear regression between the transcriptomic and transcriptional (qPCR) data (B). Immunoblot analyses in two independent replicates of photosynthetic proteins (RbcL, Rubisco large subunit; RbcS, Rubisco small subunit; Fd, ferredoxin; LHCII, light-harvesting complex II; Psba, D1 protein) extracted from two replicates of buds of ON-Crop, (ON1 and ON2), OFF-Crop (OFF1 and OFF2), and de-fruited (DEF1 and DEF2) trees at Time 4 (C). The quantification of the protein signals, generated using ImageJ software, is presented in Supplementary Fig. S4.

Two other induced genes, *Cs9g20300.1* and *Cs8g20150.1*, showed high homology (80% and 70%, respectively) to a calcium-dependent protein kinase (*At1g08650.1*), and to a Ca^{2+}/H^{+} antiporter *CAX3* (*At3g51860*), respectively. Another gene, *Cs1g21460.1*, which showed homology to members of the *NPH3* gene family from *Arabidopsis* also plays a role in auxin polar transport (Furutani *et al.*, 2011; Knauer *et al.*, 2011; Li *et al.*, 2011; Wan *et al.*, 2012). This family contains 33 genes in the *Arabidopsis* genome, and the *Citrus* genome comprises 25 homologous genes with relatively close taxonomic relationships (Supplementary Fig. S8B). The *NPH3-like* gene was induced 25- to 40-fold in OFF-Crop and DEF buds relative to ON-Crop buds at all time points tested (Fig. 8A). The plausible mechanistic relationships between calcium, PBP1, NPH3, and the polar subcellular localization of PIN-FORMED (PIN) auxin efflux carriers are schematically presented in Fig. 8B. The expression levels of *CAX-like*, *NPH3-like*, two *PBP1-like* genes, and one Ca^{2+}-binding EF-hand gene were validated by qPCR analyses (Fig. 8C). The mRNA levels of all these genes were relatively low in ON-Crop buds and remained low throughout the experimental period. In OFF-Crop buds, they were significantly higher at Time 0 and Time 4 (by factors of 5–19 and 10–28, respectively). One week after de-fruiting, the expression of all these genes was significantly increased, and within 2 weeks they attained their maximal levels. While the mRNA levels of CAX-like, NPH3-like, and Ca^{2+}-binding EF-Hand1-like remained high 4 weeks after de-fruiting, those of PBP1-like1 and PBP1-like2 were reduced by ~2-fold and 1.5-fold, respectively, during the same period.

Auxin levels are significantly higher in ON-Crop buds and decrease following fruit removal

Next, levels of endogenous IAA were examined in the buds (Fig. 9). IAA levels were significantly higher in ON-Crop buds relative to OFF-Crop buds by 2.9-fold at Time 0 and by

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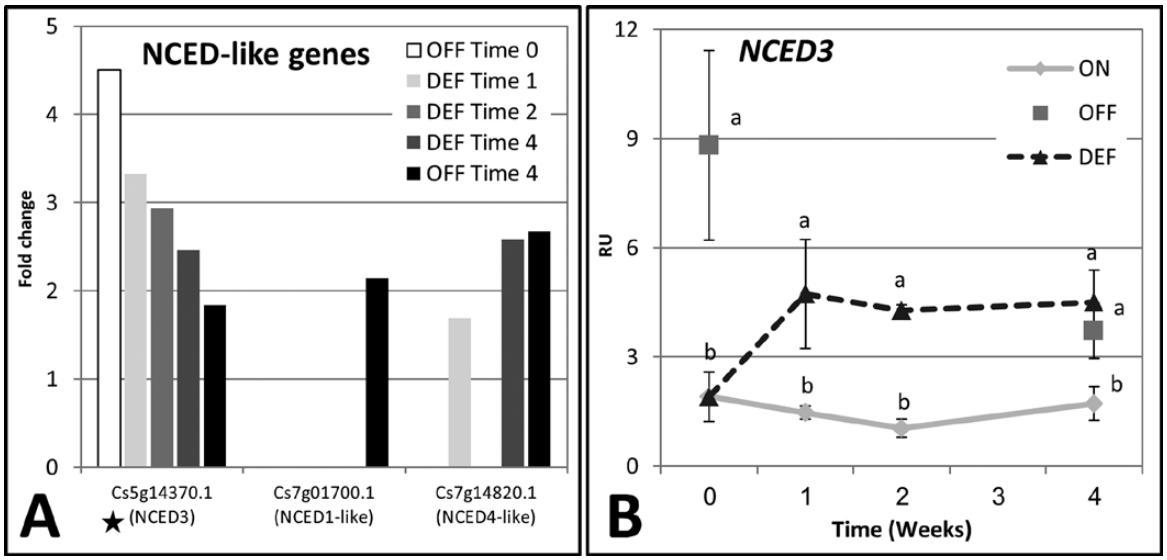

Fig. 6. *NCED*-like genes are induced in buds of de-fruited (DEF) trees. Fold change ($P \leq 0.05$) in the expression of three *NCED-like* genes in buds of OFF-Crop (OFF) and DEF trees relative to buds of ON-Crop (ON) trees (an asterisk marks the *NCED3-like* gene which was validated by qPCR) (A). Expression of the *NCED3-like* gene in buds of ON, OFF, and DEF trees as determined by qPCR analyses at the indicated weeks following de-fruiting (RU, relative units). The numbers are mean values of three independent biological replicates ±SE (B). Different letters represent a significant difference ($P \leq 0.05$) between the states at the same time point.

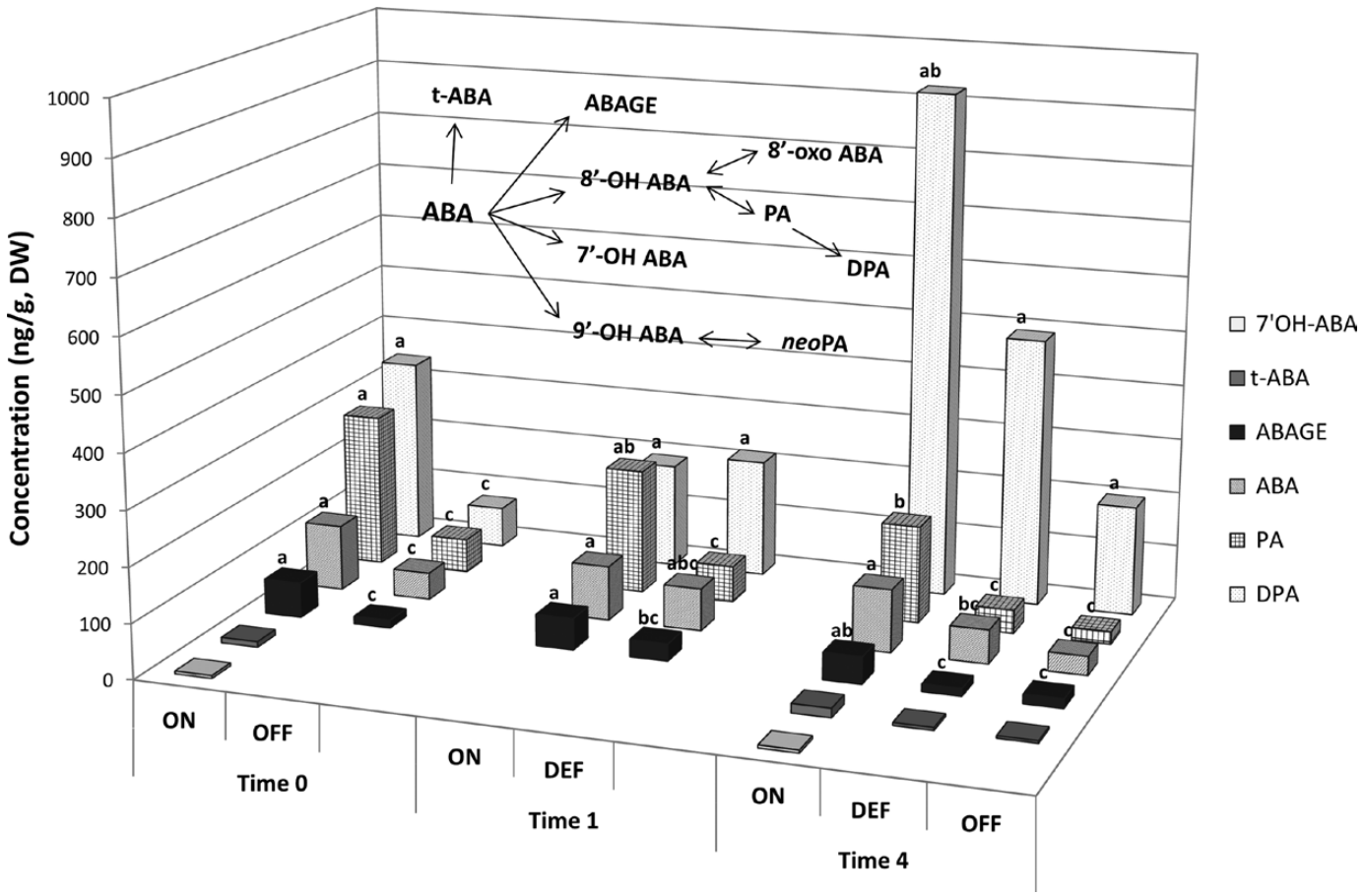

Fig. 7. Fruit load affects the levels ABA and its catabolites in the bud. ABA and ABA catabolites (7'OH ABA, t-ABA, *trans*-ABA; ABAGE, ABA glucose ester; PA, phaseic acid; DPA, di-hydrophaseic acid) whose metabolic relationships are schematically represented in the inner scheme were determined at the indicated time points in buds of ON-Crop (ON), OFF-Crop (OFF), and de-fruited (DEF) trees. Means followed by different letters are significantly different ($P > 0.05$) according to Tukey–Kramer multiple comparisons tests.

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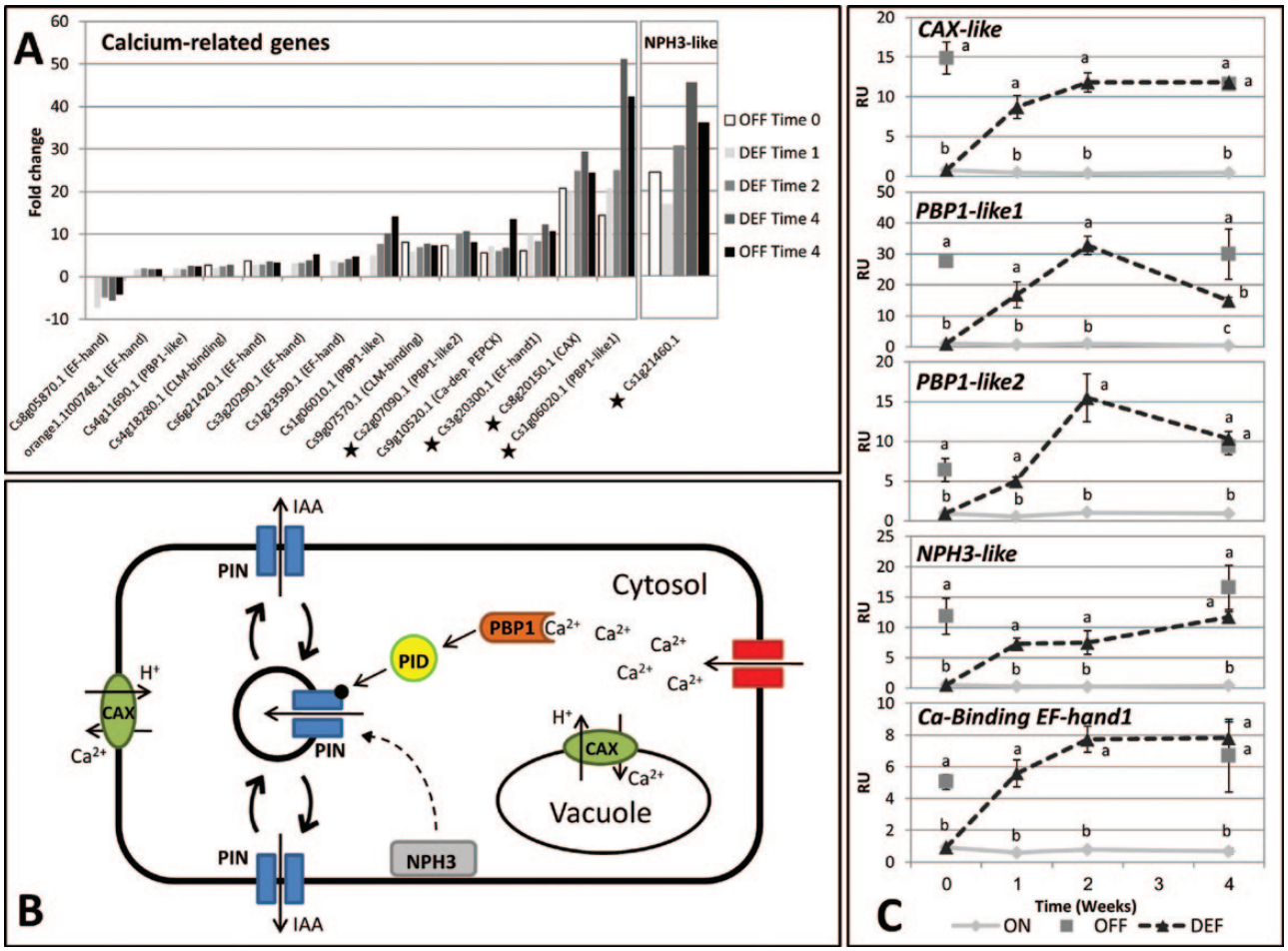

Fig. 8. Fruit load affects the expression of Ca^{2+}-related auxin polar transport and *NPH3-like* genes. Fold change ($P \leq 0.05$) in the expression of Ca^{2+}-related and *NPH3-like* genes in buds of OFF-Crop (OFF) and de-fruited (DEF) trees relative to buds of ON-Crop (ON) trees (asterisks mark genes selected for validation by qPCR analyses; all genes are listed in Supplementary File 2 at *JXB* online) (A). Expression of the indicated genes in buds of ON, OFFm and DEF trees as validated by qPCR at the indicated time points following de-fruiting (RU, relative units). The numbers represent mean values of three independent biological replicates ±SE. Different letters represent a significant difference ($P \leq 0.05$) between the states at the same time point. (B) Linear regression between transcriptomic data and transcriptional data (qPCR analyses) is presented in Supplementary Fig. S7. Schematic model representing Ca^{2+} and NPH3 regulation of PIN cellular localization (C). (This figure is available in colour at *JXB* online.)

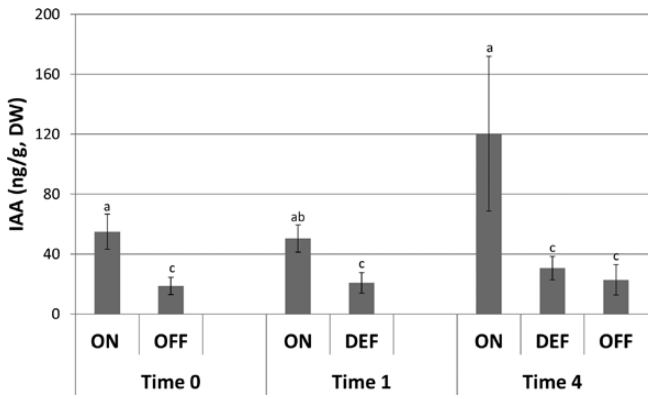

Fig. 9. Fruit load affects auxin level in buds. Auxin was determined at the indicated time points in buds of ON-Crop (ON), OFF-Crop (OFF), and de-fruited (DEF) trees. The numbers are mean values of three independent biological replicates. Means followed by different letters are significantly different ($P > 0.05$) according to Tukey–Kramer multiple comparisons tests. (This figure is available in colour at *JXB* online.)

5.2-fold at Time 4. As expected, IAA levels decreased in DEF buds relative to ON-Crop buds, by a factor of 2.4 at Time 1, and remained at this level 4 weeks after de-fruiting.

Discussion

Intensity of return bloom is affected by de-fruiting, which induces relatively rapid changes in expression of flowering control genes and in the transcriptome

Fruit removal has been reported to be effective in inducing return bloom (Monselise and Goldschmidt, 1981). However, since annual variation and cultivar-dependent divergence may affect its effectiveness (Verreynne and Lovatt, 2009; Martinez-Fuentes *et al.*, 2010; Muñoz-Fambuena *et al.*, 2011), fruit removal was carried out as early as the last 10 d of August. Indeed, the number of inflorescences and vegetative shoots, counted during the following spring (Fig. 1), showed that

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those of DEF trees were similar to those of OFF-Crop trees, thus demonstrating the effectiveness of the treatment. Although it could be expected that the number of generative inflorescences would be higher in DEF than OFF-Crop trees (Verreynne and Lovatt, 2009), the actual number was identical, probably due to the low number of fruits on OFF-Crop trees (~45-fold lower than in ON-Crop trees). De-fruiting resulted in relatively rapid changes in the expression of genes controlling and genes associated with trehalose and flavonoid biosynthesis, allowing determination of the time frame of the genomic analysis.

As a rule, the expression of flowering control genes is induced in leaves, buds, and stems in association with the onset of the flowering induction period (November–December) in regular bearer cultivars, and in AB cultivars during the OFF-Crop year, while GA treatment reduced their expression (Nishikawa *et al.*, 2007; Muñoz-Fambuena *et al.*, 2011, 2012*b*; Shalom *et al.*, 2012; Goldberg-Moeller *et al.*, 2013). *CiSPL5* is an exception to this rule, probably due to its highly regulatory role, and it exhibited higher expression in buds from May until September (Shalom *et al.*, 2012). *CiFT2* expression was usually earlier than the onset of the flowering induction period (Shalom *et al.*, 2012). Taking into account these expression patterns and the expected year to year alternation, the mRNA levels of *CiFT2* and *CiSPL5* were higher in OFF- than in ON-Crop buds. Therefore, the increase in the expression of *CiFT2* and *CiSPL5* in buds of DEF trees to levels similar to those in buds of OFF-Crop trees can be expected, and reinforces their role in return bloom. It is not surprising that during the time of the experiment, *LFY* did not show any difference in its expression between buds of ON- and OFF-Crop trees. Nevertheless, de-fruiting resulted in a 2-fold increase in its mRNA level, which returned to its basal level 4 weeks after treatment. Whether this temporary response has any relationship to return bloom requires further research.

The differences between bud populations—those with a 55% chance to flower (ON-Crop) and those with a 96% chance (OFF-Crop and DEF buds)—did not seem to be very high. However, genomic analysis resulted in numerous differentially expressed genes (DEGs), allowing the partial identification of mechanisms that convert ON into OFF buds. Previously it was shown that the number of DEGs between ON- and OFF-Crop buds was considerably lower in September than in May (Shalom *et al.*, 2012). However, the present work showed that the number of these DEGs was quite high. This difference could be explained by year to year alternations, and differences in the methodologies used. Based on a cut-off of 50% coverage between sequences on the microarray and the currently identified sequences, and on at least 75% identity, it is estimated that only ~30–35% of the current sequences are present on the microarray, supporting this notion. Only 40% of the auxin transport-related genes (Fig. 8A) were found on the microarray. Below, three identified mechanisms, common to OFF and DEF buds, which are altered during the conversion of DEF buds into OFF buds, and might play a role in the signalling mechanism of fruit load are discussed.

Induction of photosynthetic gene expression and protein levels in the bud following de-fruiting

In agreement with Shalom *et al.* (2012), this study demonstrates that de-fruiting induces expression of photosynthetic genes in the bud. Although a recent proteomic analysis did not show an increase in photosynthetic proteins in OFF-Crop trees (Muñoz-Fambuena *et al.*, 2013), here the induced gene expression resulted in increased protein levels of four major genes. According to the C/N theory, the proteins of photosynthetic machinery represent the majority of leaf nitrogen which is directly related to photosynthetic capacity (Evans, 1989); thus, the induced levels of photosynthesis proteins would suggest the induction of photosynthesis in OFF buds, although direct evidence is missing. Although bud photosynthesis was never measured in fruit trees, leaf photosynthesis in relation to fruit load has been measured in previous studies. While some workers found no change in photosynthesis between leaves of ON- and OFF-Crop trees (Roper *et al.*, 1988; Monerri *et al.*, 2011; Nebauer *et al.*, 2013), others reported increased photosynthetic and CO_2 assimilation rates in fruit-bearing as compared with non-fruit-bearing trees (Fujii and Kennedy, 1985; Dejong, 1986; Gucci *et al.*, 1995; Palmer *et al.*, 1997; Iglesias *et al.*, 2002; Syvertsen *et al.*, 2003; Urban *et al.*, 2004). Vegetative growth is induced in buds of OFF-Crop and DEF trees (Monselise and Goldschmidt, 1982), suggesting that increased photosynthesis may mark the initiation of vegetative growth. That is, due to fruit absence, the OFF-Crop and DEF trees are heavily loaded with photoassimilates, suggesting that by induction of its photosynthetic machinery, the bud signals to stop translocation of photoassimilates. The possibility that the flow of photoassimilates into the bud is reduced due to lower leaf photosynthesis in OFF-Crop trees, resulting in increased synthesis of photosynthesis proteins and higher CO_2 assimilation, cannot be excluded.

Bud ABA is reduced in OFF-Crop trees and following de-fruiting compared with ON-Crop trees

Increased expression of three *NCED-like* genes, in buds of DEF and OFF-Crop trees compared with buds of ON-Crop trees, suggests the induction of ABA biosynthesis. However, direct measurements of ABA and its catabolites showed the opposite trend, namely reduced levels in buds of OFF-Crop and DEF trees. Direct biochemical evidence demonstrated that *NCED3* (*Cs5g14370*) cleaved 9-*cis*-violaxanthin to form xanthoxin, a precursor of ABA (Kato *et al.*, 2006); its expression paralleled ABA levels in the peel and during cycles of drought and re-watering of leaves and fruit (Rodrigo *et al.*, 2006; Agustí *et al.*, 2007). Therefore, one would expect higher ABA levels in OFF-Crop and DEF buds than in ON-Crop buds. A possible explanation of these apparently contradictory results is that the source of ABA in the ON-Crop bud is not within the bud itself, but external to it, and dependent on the presence of fruit. In OFF-Crop trees or following de-fruiting, the translocation of ABA from this source into the bud is blocked, at least partially, reducing the bud's

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ABA contents, and inducing *NCDE3* expression in order to increase endogenous ABA production. Nevertheless, the possibility cannot be excluded that induced expression of *NCED* genes is futile, and has no physiological role. A closer look at ABA-responsive genes in the transcriptomic data (overall, eight differentially expressed genes between the states) did not solve this contradiction, as no common trend in their response was evident (data not shown). Regardless, ABA levels (and the expression of ABA receptor component, *PYR1-like*) in OFF-Crop buds and following de-fruiting were reduced, raising the question of its possible involvement in AB control. Consistent with the present results, buds of ON-Crop trees have been shown to contain higher levels of ABA or its isomer, t-ABA, than those of OFF-Crop trees (Jones *et al.*, 1976; Goldschmidt, 1984). It has been suggested that elevated levels of ABA in ON organs may reflect a stress imposed by the fruit overload. Moreover, ABA might serve as an inhibitor of return bloom, since the local application of ABA to *Citrus unshiu* buds in late December inhibited bud sprouting and intensive flowering (Garcialuis *et al.*, 1986). Alternatively, the possibility that flowering promotes ABA activity has been suggested, since increased ABA levels were detected in leaves of OFF-Crop trees and following de-fruiting of ON-Crop trees in association with flowering induction (Koshita *et al.*, 1999; Okuda, 2000). Whether ABA plays a role in AB control, or in other processes, such as maintaining the bud in an inactive state (Little and Edit, 1968; Horvath *et al.*, 2003; Shalom *et al.*, 2012), requires further investigation.

De-fruiting induces genes of calcium-dependent auxin polar transport

The results showed an increase in the expression of calcium-related genes together with significant reduction in auxin levels in OFF-Crop buds and in buds following de-fruiting as compared with ON-Crop buds. Changes in the concentration of cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$), mediated by ion channels, Ca^{2+}-ATPases, and Ca^{2+}/H^{+} antiporters, form the basis of the Ca^{2+} signalling mechanism. The CAX-type antiporters are a family of cytosolic low-affinity Ca^{2+}/H^{+} antiporters, which in *Arabidopsis* comprises six members. In *Citrus* there are four highly CAX homologous genes, and the expression of a CAX3 homologue was highly induced following de-fruiting. Transduction of Ca^{2+} signals is carried out by specific calcium-binding proteins, containing a common structural motif called the 'EF-hand', a helix–loop–helix structure that binds a single Ca^{2+} ion (Day *et al.*, 2002). The present results showed a significant up-regulation of a few genes encoding EF-hand proteins in OFF and DEF buds compared with their level in ON buds. Overall, these results might suggest that $[Ca^{2+}]_{cyt}$ is affected by fruit load, although at this stage a plausible scenario as to the nature of the change and its cellular signature cannot be suggested. How are these changes related to auxin polar transport? Four of the up-regulated EF-hand genes show remarkable homology to the genes encoding PBP1 in *Arabidopsis*. PBP1 interacts physically with PID protein kinase, regulating its activity in response to changes in calcium levels (Benjamins *et al.*, 2003). PID regulates the polarity of PIN proteins (Friml *et al.*, 2004), which are known to direct auxin flow (Wisniewska *et al.*, 2006). NPH3-like proteins have recently been shown to affect PIN localization (Furutani *et al.*, 2011; Wan *et al.*, 2012). As shown here, *NPH3-like* genes are part of a relatively large gene family. Divergence of its different members occurred before the divergence of *Arabidopsis* and *Citrus*. The citrus *NPH3-like* gene induced in OFF and DEF buds compared with ON buds showed very close homology to an *Arabidopsis* gene which has not yet been subjected to detailed analysis.

Taken together, the present results lead to the suggestion that higher levels of IAA in ON buds reflect their inability to distribute IAA efficiently via the Ca^{2+}-dependent PIN-based polar auxin transport mechanism. In addition, efficient auxin removal from the bud appears to be a key component in transforming the ON bud into an OFF bud. The involvement of auxin in flowering inhibition following an ON-Crop year was recently suggested (Smith and Samach, 2013), and is based on the ATA hypothesis proposed by Bangerth (Bangerth, 1989, 2006; Caaejas and Bangerth, 1997). The application of auxin polar transport inhibitors resulted in flowering induction in a number of fruit trees (Bukovac, 1968; Ben-Tal and Lavee, 1985; Ito *et al.*, 2001; Blaikie *et al.*, 2004; Bangerth, 2006). The strong polar transport of auxin from the dominant sinks (i.e. the fruit or the seed), as suggested by the ATA hypothesis, preventing auxin export from the bud, would explain why auxin levels in OFF buds and in buds following de-fruiting are lower than in ON buds.

The parallel reduction in ABA and IAA levels in the bud would suggest cross-talk between the ABA and IAA signalling pathways. Such cross-talk interactions were suggested in *Arabidopsis* embryo axis elongation and root development (Belin *et al.*, 2009; Shkolnik-Inbar and Bar-Zvi, 2010; Wang *et al.*, 2011), but not in flowering control processes.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary File 1

Figure S1. De-fruiting alters the expression of trehalose- and flavonoid-metabolizing genes.

Figure S2. Venn diagrams of developmentally regulated genes.

Figure S3. Photosynthetic genes are induced following de-fruiting in the bud.

Figure S4. Quantification of protein blot results (Fig. 5C).

Figure S5. Schematic representation of the cleavage of 9-*cis* xanthophylls to xanthoxin by 9-*cis*-epoxycarotenoid dioxygenase (NCED), a key regulated step in the biosynthesis of ABA in plants.

Figure S6. Changes in the expression of the *PYR1-like* gene.

Figure S7. Linear regression between transcriptomic and transcriptional (qPCR) data of Ca-related and *NPH3-like* genes, presented in Fig. 8A and B.

Figure S8. Genes encoding PINOID (PID)-binding protein 1 (PBP1) and Non-Phototropic Hypocotyl 3-like (NPH3) show homology in *Arabidopsis* and *Citrus*.

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Table S1. List of primers used in this study.

Table S2. List of genes used to design probes for nCounter analysis (Shalom *et al.*, 2012).

Table S3. Statistical data of deep sequencing analysis.

Table S4. GO categorization of unique genes up- or down-regulated in buds of OFF-Crop and de-fruited (DEF) trees relative to buds of ON-Crop trees during Time 4.

Table S5. GO categorization of genes of cluster 4 of DEF buds.

Table S6. GO categorization of up-regulated genes of OFF-Crop buds.

Table S7. GO categorization of down-regulated genes of OFF-Crop buds.

Table S8. GO categorization of genes of cluster 3 of DEF buds.

Table S9. GO categorization of genes of cluster 2 of ON-Crop buds.

Supplementary File 2

The file includes: (i) all possible comparisons between the treatments; (ii) the accession numbers of genes presented in the Venn diagrams (Fig. 4A, B; Supplementary S2A, B); (iii) photosynthesis genes (Fig. 5A); and (iv) calcium-related genes (Fig. 8A).

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Alternate Bearing in Citrus: Changes in the Expression of Flowering Control Genes and in Global Gene Expression in ON- versus OFF-Crop Trees

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Abstract

Alternate bearing (AB) is the process in fruit trees by which cycles of heavy yield (ON crop) one year are followed by a light yield (OFF crop) the next. Heavy yield usually reduces flowering intensity the following year. Despite its agricultural importance, how the developing crop influences the following year's return bloom and yield is not fully understood. It might be assumed that an 'AB signal' is generated in the fruit, or in another organ that senses fruit presence, and moves into the bud to determine its fate—flowering or vegetative growth. The bud then responds to fruit presence by altering regulatory and metabolic pathways. Determining these pathways, and when they are altered, might indicate the nature of this putative AB signal. We studied bud morphology, the expression of flowering control genes, and global gene expression in ON- and OFF-crop buds. In May, shortly after flowering and fruit set, OFF-crop buds were already significantly longer than ON-crop buds. The number of differentially expressed genes was higher in May than at the other tested time points. Processes differentially expressed between ON- and OFF-crop trees included key metabolic and regulatory pathways, such as photosynthesis and secondary metabolism. The expression of genes of trehalose metabolism and flavonoid metabolism was validated by nCounter technology, and the latter was confirmed by metabolomic analysis. Among genes induced in OFF-crop trees was one homologous to *SQUAMOSA PROMOTER BINDING-LIKE* (*SPL*), which controls juvenile-to-adult and annual phase transitions, regulated by *miR156*. The expression pattern of *SPL*-like, *miR156* and other flowering control genes suggested that fruit load affects bud fate, and therefore development and metabolism, a relatively long time before the flowering induction period. Results shed light on some of the metabolic and regulatory processes that are altered in ON and OFF buds.

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Introduction

Alternate bearing (AB) is the process by which cycles of heavy yield (ON crop) one year are followed by a light yield (OFF crop) the next (reviewed in [1]). AB occurs in both deciduous and evergreen fruit and nut tree crops and in forest trees (where it is called "masting"), regardless of their annual reproductive and vegetative cycles. Although in general, the ON and OFF cycles are biennial, in some cases an ON year can be followed by two or more consecutive OFF years, and vice versa. In the classical, most common AB, the OFF year is characterized by low floral intensity (reduced flower number), resulting in low yield, and high vegetative shoot growth, whereas the opposite occurs during the ON year. In some cases, flowering is not limited, but heavy flower and/or fruitlet drop lead to AB. Synchronization among different trees at the plantation/region level is typically initiated by environmental conditions (such as low and high temperatures, water deficit, etc.) that reduce yield. Once initiated, AB becomes entrained through the effect of crop load on endogenous tree factors that ultimately impact the floral intensity; the heavy ON crop reduces return bloom the following spring, whereas the light OFF crop results in an intense return bloom the following spring. Fruit thinning or complete removal (defruiting) as late as September to December of the ON-crop year induces flowering and yield in the following year [2–5]. AB has significant economic consequences in many important tree crops. In citrus, during the low-yield OFF year, a significant proportion of the fruit are too large. During the ON year, many small-size fruit with low commercial value are produced.

The mechanism(s) by which the developing crop influences return bloom and yield the following year is not fully understood. Two hypotheses have been suggested. The "nutritional" hypothesis holds that return bloom and yield are proportional to tree carbohydrate status. Lack of carbohydrate in the ON year directly or indirectly reduces flowering the following year [6]. Support for this hypothesis has been provided by showing positive correlations between carbohydrate levels and AB status [7–13], whereas others have shown no consistent relationship between tree carbohydrate

status and floral intensity at return bloom [2,3,14–19]. The ''hormonal'' hypothesis proposes that developing fruit produce an inhibitor that directly or indirectly reduces flowering in the spring following the ON crop [20–22]. Although a number of studies have shown correlations between abscisic acid or indole-3-acetic acid and AB status [4,13,23–26], no direct evidence has been provided for their involvement in the return bloom. Gibberellin (GA) is well-known inhibitor of flowering in citrus; thus, fruit-produced GA has been presumed to be involved in AB [27,28]. Despite these findings, the roles of carbohydrates and hormones in AB remain unclear and more research is needed to identify factors affecting floral intensity following ON and OFF years. Genetic analysis of AB in apple identified a few QTLs associated with AB, and suggested that hormone-related genes are likely to play a role in the phenomenon [29].

The floral induction period in citrus starts in mid-November and lasts until approximately the end of December to mid-January (Figure S1, the annual cycle in citrus) [30]. Following induction, the bud enters a short resting period, after which the shoot apical meristem differentiates into a floral bud [31,32]. In parallel to the floral shoot flush, there is a flush of vegetative shoot growth (Figure S2), which continues through June (Spring flush). A second flush of vegetative shoot growth starts in July (Summer flush), and third flush starts in October (Fall flush). Usually, next year flowering occurs mostly on the spring vegetative flush [33]. Flowering in citrus is induced by low temperature, while day length has a relatively minor effect [30]. There is extensive cross-talk between autonomous and vernalization flowering pathways and ample evidence that genes associated with flowering regulation are highly conserved across species [34]. Indeed, citrus genes homologous to *Arabidopsis* flowering control genes most likely possess similar functions. For instance, overexpression of the citrus *FLOWERING LOCUS T* (*FT*), arabidopsis *LEAFY (LFY)* and arabidopsis *APETALA1* (*AP1*) genes in citrus greatly reduced the juvenile period, allowing flowering at the seedling stage [35–37]. *FT* was shown to be induced during the annual transition to floral development [38]. In addition, *FT* transcript accumulated in trees subjected to low-temperature floral-inductive conditions [38]. Overexpression of the citrus *LFY*, *AP1* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) genes in *Arabidopsis* resulted in phenotypes similar to those observed when the endogenous genes were overexpressed, and *CsLFY* and *CsAP1* rescued *Arabidopsis* mutants in the respective genes [34,37]. Similar findings were demonstrated for the citrus *TERMINAL FLOWER* homolog (*CsTFL*) [39]. Inverse relationships were found between fruit load and the expression of *FT, AP1* and *SOC1* in the leaves of 'Moncada' mandarin, especially during the flowering induction period [40].

Fruit presence inhibits return flowering. However, it is not clear at which stage the fruit exerts its inhibitory effect: at flowering induction, transition of the shoot apical meristem to floral meristem, or subsequent stages of floral development and bud break. Moreover, the nature of the signal ('AB signal') and the organ or tissue from which it originates, be it the fruit itself or the leaf which senses fruit presence, are not known. Regardless of the source tissue for the AB signal, it must be received, directly or indirectly, at the bud, and more specifically, at the apical meristem which has to ''decide'' whether to develop into an inflorescence or remain a vegetative meristem. Therefore, following perception of the signal, the bud must undergo a series of events which depend on fruit load. In the current work, we analyzed changes in global gene expression during bud development in ON and OFF trees, to identify metabolic and controlling pathways that play a role in bud fate. To determine the earliest time point for the transcriptome analysis, we first analyzed changes in bud morphology during its development, and changes in the expression of key flowering control genes. Based on those results, global gene-expression analysis was carried out at a few key time points in the buds, which receive the 'AB signal', and in leaves and stems, which might play a role in generating and transporting the signal.

Materials and Methods

Plant material

Plant material was collected from a commercial orchard of 10-year-old Murcott mandarin (*Citrus reticulate* Blanco) trees grafted on sour orange (*Citrus aurantium* L.), located in the central coastal area of Israel, during the years 2009 (an OFF year) and 2010 (an ON year). Although most of the trees in the orchard yielded similarly in a given year, some were exceptional and showed an opposite AB trend. These and nearby trees with the opposite yield status were selected. Overall, nine pairs of trees were chosen, with each three pairs (ON tree and nearby OFF tree) being considered one biological replicate. Comparisons included the two most extremes conditions in regards to chance to flower of buds on the spring flush (Figure S2): fruit-bearing flush of an ON tree and fruitless flush of an OFF tree. About 10 fruitless branches from OFF trees and about 25 fruit-bearing branches from ON trees (Figure S2), collected from the southeast side of the tree, were taken to the laboratory on ice. Leaves and stems and at least 10 buds were removed from the 2 to 3 most distal nodes of one OFF fruitless spring flush (Figure S2) and immediately frozen in liquid nitrogen. Leaves and stems and all buds of a fruit bearing ON spring flush (Figure S2) were removed and immediately frozen in liquid nitrogen. Samples were kept at $-80^{\circ}C$ until processing. For the genomic analyses, the collections of leaves, stems and buds was as the following in regards to fruit development (Figure S1): mid-May stage I [41], mid- July, early stage II and mid-September, late stage II. For gene expression analyses, samples were collected during the middle of the indicated month. The numbers of inflorescences and vegetative shoots were determined for all of the branches splitting from one major 50- to 60-mm diameter branch located on the southeast side of the tree, during peak blossom, usually the first third of April of the consecutive year. Selection of the sampled branch was performed prior to bud break.

Light microscopy

Buds were collected and fixed in an FAA solution [10 formaldehyde:5 acetic acid:85 ethanol (70%), v/v]. Fixation was followed by an ethanol dilution series and subsequent stepwise exchange of ethanol with Histoclear (xylem substitute). Samples were embedded in paraffin and cut by microtome (Leica RM2245) into 12-μm sections. Sections were stained with safranin and fast green [42], and examined under a light microscope (Olympus BX50, 50–100× magnification).

RNA extraction and gene-expression analysis by real-time PCR

Total RNA was extracted from buds and from leaves and stems (LS) using the CTAB extraction method [43]. For buds, approximately 0.2 g of frozen tissue was used, and approximately 2 g of tissue for LS. The volumes of the extraction solutions were adjusted to the amount of starting material. RNA was treated with RQ1 RNase-free DNase (Promega, Fitchburg, WI) according to the manufacturer's instructions. RNA quantity was analyzed in a NanoDrop ND-1000 Spectrophotometer (Wilmington, DE) and RNA quality was determined by Agilent Bioanalyzer (Santa Clara, CA). cDNA was synthesized from 1 μg RNA using OligoT as a

primer and M-MLV Reverse transcriptase (Fermentas, Burlington, Ontario, Canada) in a final volume of 25 µl containing the commercially supplied buffer. Primers for the genes *CiFT1/2/3*, *CsAP1*, *SOC1*, *CsLFY*, *β-actin*, and dual-labeled probes for *CiFT1/2/3* were designed based on genomic and EST sequences (Phytozome, http://www.phytozome.net/, HarvEST, http://harvest.ucr.edu/) using Primer 3 software (Table S1). For the SYBR green reactions, real-time PCR was carried out in a reaction mix containing 2 µM gene-specific forward and reverse primers, 3 µl cDNA (diluted 1:16), KAPA SYBR FAST qPCR Master Mix (2×) Universal (KAPA Biosystems, Boston, MA), and Ultra-Pure water (Fisher Biotech, Wembley, Australia) in a final volume of 12 µl in a Corbett Rotor-Gene 6000 (Qiagen, Venlo, The Netherlands). Reactions were run for 40 cycles of 10 s at 95°C, 15 s at the annealing temperature for each gene, 20 s extension at 72°C, and the threshold level was determined. For the dual-labeled probe reactions, real-time PCR was carried out in a reaction mix containing 2 µM gene-specific forward and reverse primers, 2.5 µM dual-labeled probes, 3 µl cDNA (diluted 1:16), TaqMan Universal PCR (2×) Master Mix (Applied Biosystems, Inc., Foster City, CA) and Ultra-Pure water in a final volume of 12 µl in the Rotor-Gene 6000. Reactions were run for 40 cycles of 15 s at 95°C, 60 s annealing and extension at 60°C, and the threshold level was determined. Standard curves were generated for each gene using serial cDNA dilutions. Relative concentration of the product was calculated by the algorithm of the Rotor-Gene software using the CT value. Relative expression (RE) was defined as the ratio between the relative concentration of each gene and that of *β-actin*. The expression of *miR156* was determined using TaqMan® Small RNA Assay Kit (Applied Biosystems) according to manufacturer's instructions; 10 ng total RNA was used, and real-time PCR was run in the Rotor-Gene 6000. The results were normalized against *β-actin*.

nCounter analysis

The RNA levels of trehalose biosynthetic genes, flavonoid biosynthetic genes, *SQUAMOSE PROMOTER BINDING*-like (*SPL*-like) gene and the reference genes, *β-actin*, *cyclophilin* and *polyubiquitin 2*, were determined by nCounter analysis (Nanostring Technologies, Seattle, WA) at VIB MicroArrays Facility (Leuven, Belgium) according to the manufacturer's instructions [44]. Probe design was based on genomic sequences (http://www.phytozome.net/, Table S2).

Microarray hybridization analysis

For global gene expression, the citrus GeneChip (Affymetrix, Inc., Santa Clara, CA) carrying 30,171 probes was used. The array is estimated to represent about 15,500 genes. RNA samples were processed as recommended by the Affymetrix GeneChip Expression Analysis Technical Manual at the Center for Genomic Technologies of the Hebrew University of Jerusalem. Total RNA was quantified and then adjusted to a final concentration of 1 µg/µl. Single-stranded and then double-stranded cDNA was synthesized from total RNA (0.5 µg total RNA for each reaction) using oligo-dT primer and the Affymetrix One-Cycle Labeling Kit and control reagents. The resulting double-stranded cDNA was column-purified and then used as a template to generate biotin-tagged cRNA from an *in-vitro* transcription reaction performed with the Affymetrix GeneChip IVT Labeling Kit. The resulting biotin-tagged cRNA (15 µg) was fragmented into strands of 35 to 200 bases in length following published protocols (Affymetrix GeneChip Expression Analysis Technical Manual) and then hybridized at 45°C with rotation for 16 h (Affymetrix GeneChip Hybridization Oven 320) with the Affymetrix Citrus Genome array. The arrays were washed and then stained (EukGE-WS2v5 protocol, p 2.3.11) using SAPE and biotinylated anti-SA in an Affymetrix Fluidics Station 450 followed by scanning in a GeneChip Scanner 3000. Hybridizations were carried out in triplicate, each replicate representing one experimental block. Data processing, including signal analyses, normalization and background subtraction, were carried out using Robust Microchip Analysis (RMA), as described previously [45]. Statistical test for significantly differentially expressed probes was carried out with the Linear Model for Microarray (limmaGUI) as described previously [46].

Gene ontology (GO) analysis was performed using the AgriGo interface (http://bioinfo.cau.edu.cn/agriGO/index.php). Singular enrichment analysis (SEA), which lists enriched GO terms, was used. Differentially expressed probe (DEP) sets were displayed on diagrams of metabolic and other processes using MapMan [47].

Flavonoid content analysis

Buds (200 to 300 mg) were pulverized in liquid nitrogen using a mortar and pestle and the powder was transferred to a 15-ml tube. Three volumes of water-saturated n-butanol were added, and the mixture was vortexed for a few minutes, then incubated under shaking (200 rpm) for 12 h at room temperature. Following short centrifugation (15,000 RPM at room temperature) and phase separation, the upper phase was collected into a fresh tube, and incubated at room temperature for 1 h to allow the butanol to evaporate. Samples were filtered through a Millex-HV Durapore (PVDF) membrane (0.22 µm) before injection into the LC-MS instrument. MS analyses were carried out by the ultraperformance LC-quadrupole time of flight (UPLC-QTOF) instrument (Waters Premier QTOF, Milford, USA), with the UPLC column connected on-line to a PDA detector (Waters Acquity), and then to the MS detector equipped with an electrospray ion (ESI) source (performed in ESI-positive mode). Separation was performed on a 2.1×50 mm i.d., 1.7-µm UPLC BEH C18 column (Waters Acquity). The chromatographic and MS parameters were as follows: the mobile phase consisted of 0.1% formic acid in water (phase A) and 0.1% formic acid in acetonitrile (phase B). The linear gradient program was: 100% to 95% A over 0.1 min, 95% to 5% A over 9.7 min, held at 5% A for 3.2 min, then returned to the initial conditions (95% A) in 4.2 min. The flow rate was 0.3 ml/min, and the column temperature was kept at 35°C. Masses of the eluted compounds were detected with a QTOF Premier MS instrument. The following settings were applied during the UPLC-MS runs: capillary voltage of 3.2 kV, cone voltage of 30 eV, collision energy of 5 eV, and argon as the collision gas. The following settings were applied during the UPLC-MS/MS run: capillary spray of 3.2 kV, cone voltage of 30 eV, collision energies of 15 to 25 eV, and argon as the collision gas. The m/z range was 70 to 1,000 D. The MS system was calibrated using sodium formate, and Leu-enkephalin was used as the lock mass. MassLynx software version 4.1 (Waters Inc.) was used to control the instrument and calculate accurate masses.

Statistical analysis

ANOVA test for qPCR results, bud measurements and metabolomic data was conducted using the JMP® version 10 software (SAS Institute Inc. Cary, NC).

Results

Flowering intensity and bud size are affected by fruit load

Normally, fruit load status in an AB variety is similar among most of the trees in an orchard in a given year, i.e., most trees either bear a heavy crop (ON-crop year) or a low crop (OFF-crop year). A few trees, however, show the opposite trend, allowing the collection of samples from both AB states from nearby trees. Buds, leaves and stems of heavy-loaded and low-loaded Murcott trees from the same orchard were collected from May, soon after fruit set, until January, the end of the flowering induction period. Flowering intensity of these trees was assessed the following spring (Figure 1). Citrus bears three types of inflorescences: generative (leafless), mixed (leafy, flowers and leaves at various ratios) and vegetative. As expected, in the following spring, ON-crop trees had significantly less generative inflorescences (80% vs. 15%) and more vegetative shoots (65% vs. 5%) than OFF-crop trees. No difference was detected in mixed-type inflorescences. Fruit counting during harvest time showed that ON trees yielded 232±33 fruits/tree, while OFF trees yielded 1542±102 fruits/tree. Buds were measured during the collection period using light microscopy. Usually, there were two adjacent buds in the same position (Figure 2A). External width and height measurements of buds from ON- and OFF-crop trees showed that bud height is slightly induced from May until September, with no difference between ON and OFF-crop buds (Figure 2B). Bud width did not change significantly from May until January, but OFF-crop buds were already significantly larger than ON-crop buds in May. Microscopic analyses of buds from May to January showed no structural differences, with each pair of buds having its own meristem and leaf primordia (not shown).

Seasonal changes in the expression of flowering genes in buds of ON- and OFF-crop trees

The mRNA levels of key flowering genes were measured in buds of ON- and OFF-crop trees at a few time points: mid-May—immediately after fruit set, mid-July—1 month after natural fruit thinning (June drop), and mid-September—the last time point at which fruit removal during an ON-year reverses the AB trend. In addition, samples were collected from mid-November until mid-January, considered the flowering induction period (Figure 3). The following genes were selected (genes names are in accordance with the original work in which they were functionally characterized): *CiFT* [35], *CsAP1*, *CsLFY* [37] and *SOC1* [34]. Three *CiFT* genes were analyzed. Originally, the expression of three transcripts of *CiFT* were characterized, *CiFT1*, *CiFT2* and *CiFT3*, based on the EST database [38]. However, when comparing the sequences of these three ESTs to the full genome sequence of citrus (http://www.phytozome.org/), it became evident that *CiFT1* and *CiFT2* are most likely encoded by a single gene (*Clementine0.9_023420*), while *CiFT3* is encoded by a different one (*Clementine0.9_033594*) [48]. In addition to these two genes, another gene, highly homologous to *FT*, was found in the genome sequence (*Clementine0.9_023363*) with no representative in the EST database. In the current work, the transcript of *Clementine0.9_023420* is denoted *CiFT1*, that of *Clementine0.9_033594* is denoted *CiFT2* and that of *Clementine0.9_023363* is denoted *CiFT3*.

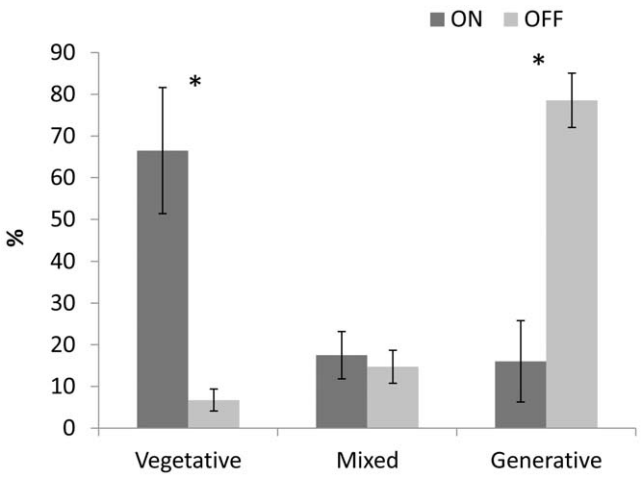

Figure 1. Effect of ON- and OFF-crop years on flowering intensity. Vegetative shoots, generative inflorescences containing only flower buds, and mixed inflorescences containing flowers and leaves, were counted during flowering peak in trees which carried heavy yield (ON) and light yield (OFF) during the previous year. Mean number of three biological replicates ± SE. Stars denote a significant difference between ON and OFF buds ($P<0.05$).
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The mRNA levels of *CiFT1* were significantly induced in ON and OFF buds from May to July, decreased toward September, and remained relatively low during the flowering induction period until January. During May and July, ON buds displayed higher transcript levels than OFF buds. The mRNA levels of *CiFT2* in buds of OFF-crop trees showed a gradual increase of 35-fold overall from September to January. Although gene expression of *CiFT2* in buds of ON trees showed a similar pattern, it was significantly lower than in OFF buds during this period. The expression of *CiFT3* in OFF buds was relatively low and did not change during the tested period. However, in ON buds, it was induced about 10-fold from July to September, and then decreased to levels similar to those of OFF buds from November until January (Figure 3).

In May, the transcript levels of *CsAP1* were about 2-fold higher in buds of ON trees than OFF trees. From July until September, the transcript levels were quite similar in buds from both tree types but, as expected, during the induction period, from November until January, there was a ca. 2.5-fold induction in transcript levels in OFF buds, whereas no such induction was detected in ON buds (Figure 3).

The expression of *SOC1* showed a very similar pattern throughout the tested period in ON and OFF organs. Buds of OFF trees showed a ca. 2-fold increase in mRNA levels relative to ON buds. However, transcript levels were reduced to minimal levels in September, and were then induced about 4-fold in both ON and OFF buds until December, followed by a small reduction toward January (Figure 3).

The expression of *CsLFY* in OFF buds fluctuated during the tested period, with a ca. 4–5-fold increase from December to January. ON buds displayed relatively constant transcript levels during the tested period (Figure 3).

Changes in global gene expression in ON vs. OFF trees

Rationale of the sampling. The above results showed that there was already a clear difference in the sizes of ON and OFF buds in May. Moreover, the mRNA levels of four key flowering control genes,*CiFT1*, *CsAP1*, *SOC1* and *CsLFY*, showed significant differences between ON and OFF buds at that time point. These results thus suggested that changes in metabolic and regulatory pathways between organs of ON and OFF trees can be expected in as early as May, soon after flowering and fruit set. Therefore, global gene-expression analysis was carried out using RNA extracted in mid-May from ON and OFF buds, as the organ

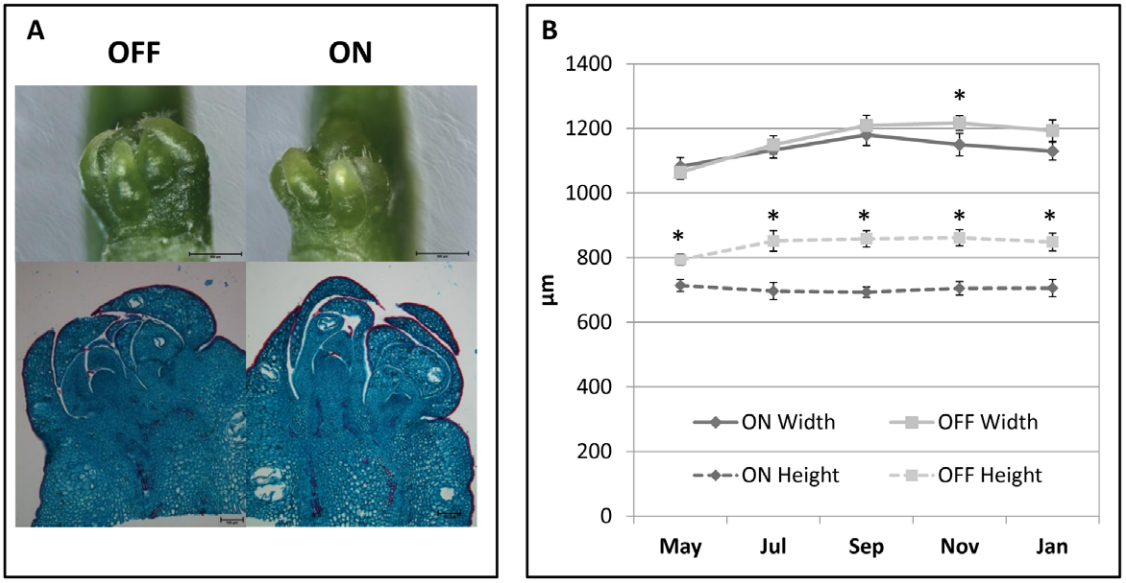

Figure 2. Bud morphology in ON- and OFF-crop trees. Buds were collected from ON- and OFF-crop trees in mid-July (A) and during the indicated months (B), fixed, dissected, dyed and photographed. Bud width and length were measured following photography (B). Mean values of 50 buds ± SE. Stars denote a significant difference between ON and OFF buds during the same time point ($P<0.02$).
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which receives the AB signal, and from pooled RNA of leaves and stems (LS), presumably playing a role in generating and transporting the AB signal. We also included an additional time point, mid-September, using RNA from the above organs. Assuming that by the induction period (November through January), the AB signal has already been generated, pooled RNA extracted from buds in December and January was included in the analysis.

Hierarchical cluster analysis and statistical analysis of hybridization results. Transcriptome analysis was carried out with the above samples using the Citrus Genome Array (Affymetrix) containing 30,171 probes and estimated to represent about 15,500 genes (Table S3). Hierarchical cluster analysis (Figure 4A) showed that the highest level of similarity in the transcription profile was in the same organ between its ON and OFF states at the same time point; different organs at the same time point showed higher levels of similarity in their transcription profiles than the same organs at different time points. Moreover, when comparing the number of DEPs ($P\leq0.05$) between buds and LS (regardless of time point and AB state), between ON and OFF states (regardless of time point and organ), and between May and September (regardless of AB state and organ), the highest number was found in the comparison of dates only (15,059); it was lower when comparing tissues only (12,770), and lowest when comparing AB states only (819).

As a comparison of the two AB states was the main target of this study, the number of DEPs in ON and OFF buds and ON and OFF LS at the various time points is presented in Figure 4B. Overall, the highest number of DEPs was detected in May for both buds and LS (6222), while much lower numbers of DEPs were found for buds and LS in September and December–January (263 and 165, respectively). Buds in May displayed the highest number of DEPs: 2205 probes with higher expression in the ON year (of which 510 displayed at least a 2-fold change) and 3087 probes with higher expression in the OFF year (of which 506 displayed at least a 2-fold change). For LS in May, 531 DEPs were found in the ON year (of which 256 displayed at least a 2-fold change) and 399 DEPs in the OFF year (58 displaying at least a 2-fold change). Only 1 and 2 DEPs were found in September buds during ON and OFF years, respectively, whereas the numbers during the induction period were 58 and 107 for ON and OFF years, respectively. In September LS, 42 and 218 DEPs were found in ON and OFF years, respectively. In searching for probes which were significantly ($P\leq0.05$) induced in OFF trees, in association with flowering induction, regardless of time or tissue, the probe *Cit.6595.1.s1_at* displayed the highest differential expression. As presented below, this probe was homologous to *SPL* transcription factor from *Arabidopsis*.

Specific pathways which are altered in ON and OFF trees in May

Considering the high number of DEPs in May relative to the other time points, GO and other analyses were performed only for ON and OFF trees in May. Overall, 1767 (out of 2205) and 2359 (out of 3087) DEPs induced in the ON year and OFF year, respectively, were GO annotated (Table S4). SEA was performed on those probes showing at least a 2-fold change. In addition, all induced and reduced probes in the buds were analyzed by MapMan for altered metabolic and regulatory pathways.

Processes induced in OFF trees relative to ON trees. None of the biological processes which were induced in OFF LS could be identified by SEA—only those in the buds were (Table 1). In buds, major enriched processes included pathways of secondary metabolism, phenylpropanoid, flavonoid and alkaloid metabolisms, and processes related to light and irradiation responses (including red/far red light) and photosynthesis. Analysis of induced and reduced probes by MapMan confirmed these results, as shown in the general metabolism scheme (Figure 5, Table S5). For secondary metabolism, most of the altered probes, belonging to terpene, flavonoid and phenylpropanoid metabolisms, were induced in the OFF buds. Metabolic pathways for some amino acids, such as serine, glycine, and cysteine, were also induced in the OFF buds. Strikingly, most of the probes belonging to the photosynthetic pathways, light reactions, photorespiration

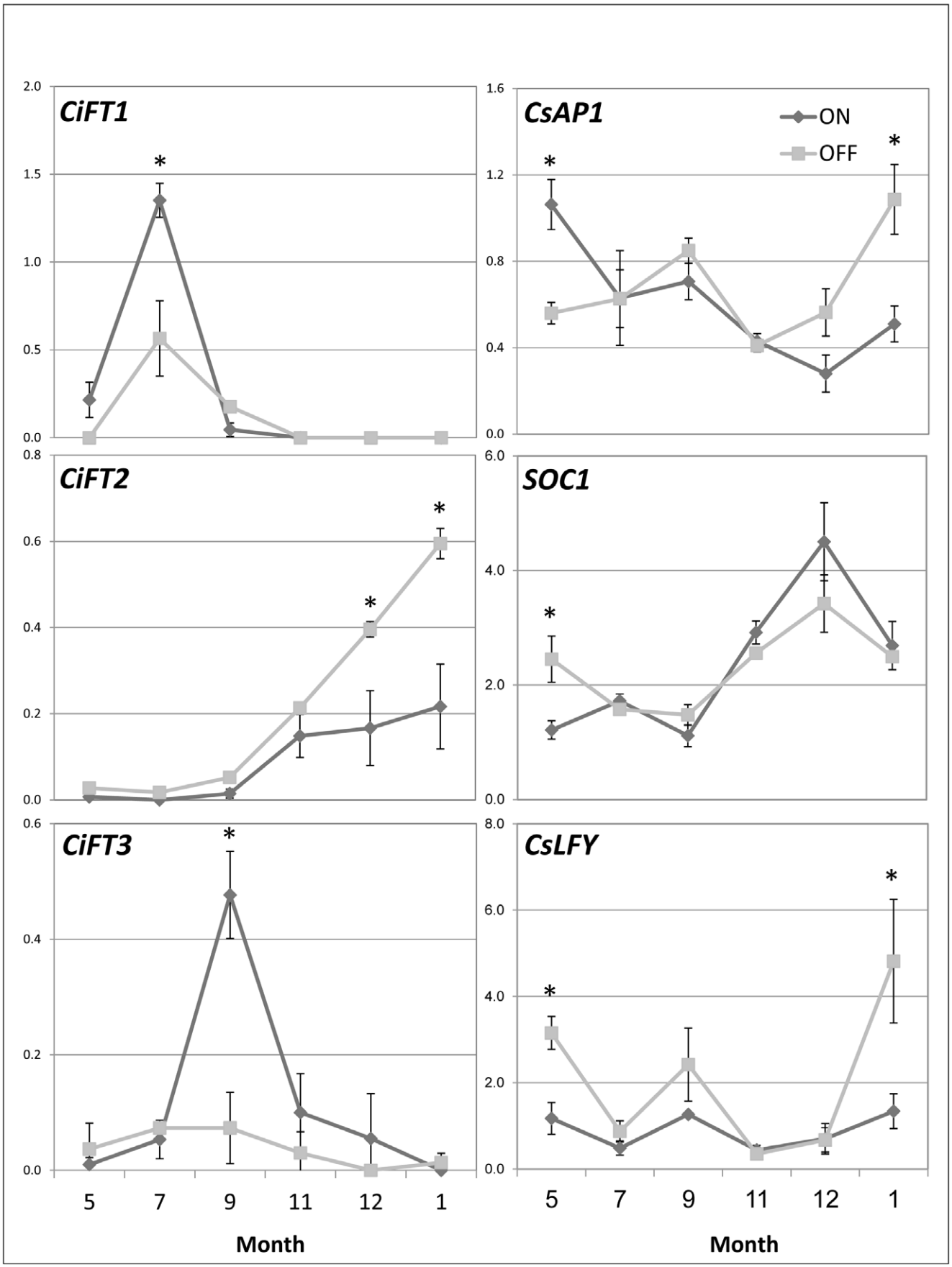
CiFT1
CsAP1
ON
OFF
CiFT2
SOC1
CiFT3
CsLFY
5
7
9
11
12
1
Month
Month

Figure 3. Differences in flowering control genes in ON vs. OFF buds. mRNA levels of the indicated genes in ON and OFF buds were determined by real-time PCR during the indicated months. Mean values of three independent biological replicates ± SE. Stars denote a significant difference between the expression of the gene in ON and OFF buds during the same time point ($P<0.05$).
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and Calvin cycle were significantly induced as well (Figure S3, Table S6). For starch metabolism, the picture seemed more complex, as both probes belonging to starch catabolism and synthesis were induced (Figure 5). Probes for the TCA cycle were moderately induced.

Processes induced in ON trees relative to OFF trees. Only one process was induced in LS during the ON year—Cell Wall Organization (GO:0009664)—which showed about 18-fold enrichment ($P = 2.4E-7$; false discovery rate = 0.0012). Similarly, in ON buds, the metabolism of glucan, a cell-wall component, showed induction (Table 2, Figure 5, Table S5). Other processes induced in the ON buds were involved in disaccharide and polysaccharide metabolism (Table 2), including trehalose and sucrose metabolisms (Figure 5, Table S5).

Expression of citrus *SPL*-like and *miR156*

As already mentioned, an *SPL*-like probe showed the highest induction level in OFF vs. ON LS and buds at all tested time points. *SPL* genes make up a family of transcription factors which have been previously shown to affect flowering time and phase change in *Arabidopsis* [49]. The citrus *SPL*-like gene showed the highest homology to *SPL3*, *SPL4* and *SPL5* from *Arabidopsis*. Members of the *Arabidopsis SPL* gene family contain a *miR156*-binding site, and direct evidence has been provided that *miR156* represses the expression of some of them [50]. A putative *miR156*-binding site was present in the 3′UTR of the citrus *SPL*-like gene. Expression of the citrus *SPL*-like gene was analyzed in ON and OFF buds throughout the year using nCounter technology (Figure 6). In OFF buds, its expression was reduced from May until the induction period. As expected, the expression in OFF buds was significantly higher than in ON buds from May to December. The expression pattern of *miR156* was also investigated in ON and OFF buds. No difference was detected between them, but the expression was slightly reduced from May until September and then induced from September and throughout the flowering induction period, in correlation with the repression in *SPL*-like gene expression.

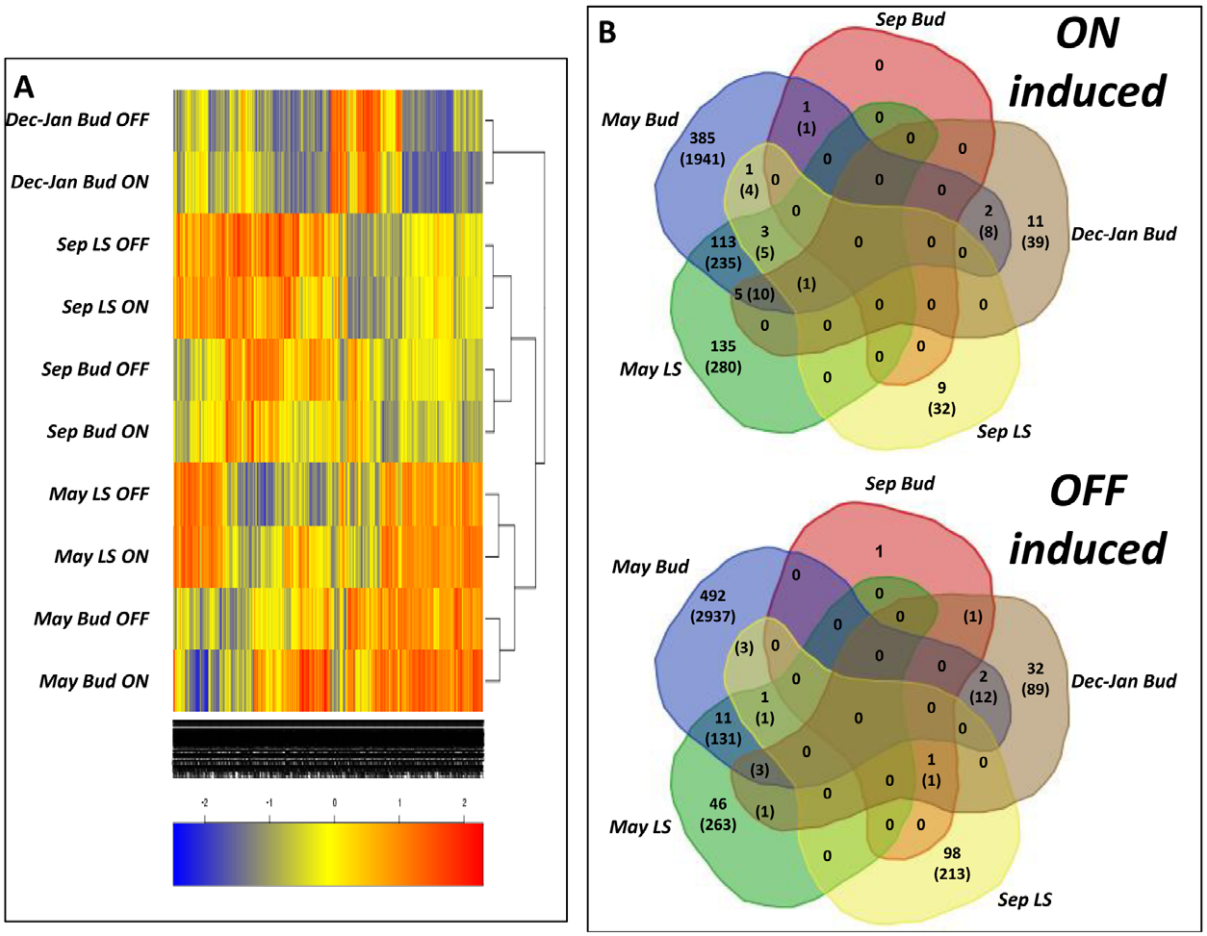


Figure 4. Differences in global gene expression in ON- vs. OFF-crop trees. Hierarchical cluster analysis of global gene expression in buds and leaves+stems (LS) in ON-crop (On) and OFF-crop (Off) trees at the indicated times (A). Venn diagrams of differentially expressed probes, induced in buds and LS of ON- and OFF-crop trees during the indicated months.
doi:10.1371/journal.pone.0046930.g004

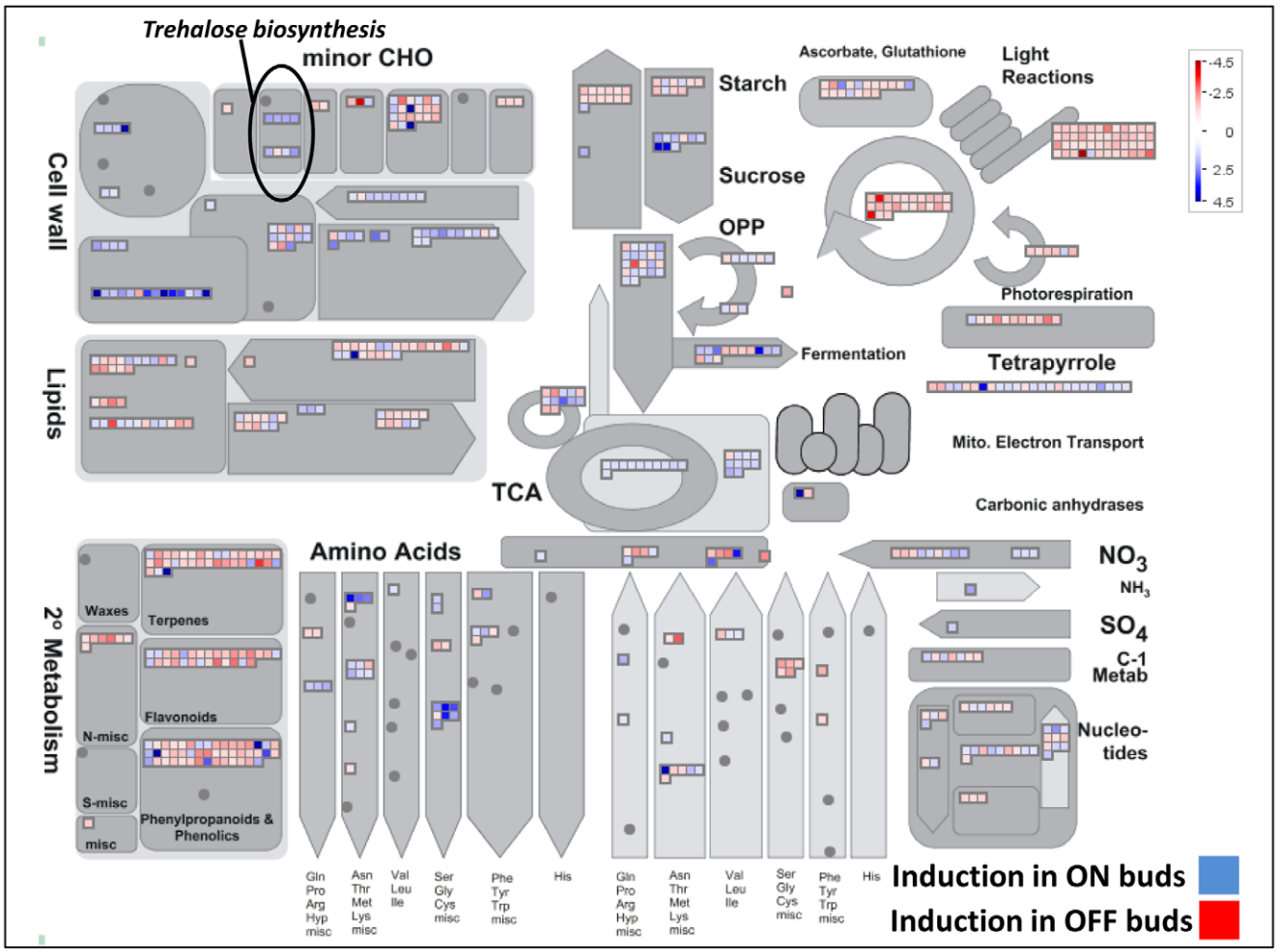


Figure 5. General metabolism in ON and OFF buds. Differentially expressed probes were analyzed using MapMan. Blue squares represent genes induced in ON buds and red squares represent genes induced in OFF buds. A description of the specific genes and their fold change is provided in Table S5.
doi:10.1371/journal.pone.0046930.g005

Expression analyses of genes of trehalose and flavonoid metabolisms, and metabolomic analyses of flavonoids

Global gene-expression analysis showed that probes encoding trehalose metabolism enzymes are induced in ON buds (Figure 7, lower panel). Validation of the microarray results by nCounter technology revealed that the two genes of trehalose metabolism, encoding trehalose phosphate phosphatase (*TPP*) and trehalose phosphate synthase (*TPS*), are indeed induced in ON buds in May, although the fold-change was lower than that detected for their corresponding microarray probes (Figure 7, upper panel). During the following months, no significant change in these two genes' expression was detected between ON and OFF buds, and their pattern of expression was different, especially from September to January (Figure S4).

Probes for six genes of the flavonoid metabolic pathway, 4-coumarate:coenzyme A ligase (*4CL*), chalcone synthase (*CHS*), chalcone isomerase (*CHI*), isoflavone reductase (*IFR*) flavonol synthase (*FLS*), and UDP-glucose:flavonoid-3-O-glucosyltransferase (*UF3GT*) were induced in OFF buds in May, whereas the probe for one gene, cinnamate 4-hydroxylase (*C4H*), was reduced in these buds (Figure 8, lower panel). Validations were therefore carried out for the 11 genes of the flavonoid biosynthetic pathway (Figure 8, upper panel). As expected, genes encoding 4CL, CHS, CHI and UF3GT showed significantly higher expression (more than 2-fold) in buds of OFF trees as compared to those of ON trees. One gene, *IFR*, showed only marginal induction in OFF buds, as compared to 2-fold induction in the microarray results. The analysis also confirmed the transcriptome analysis result that the gene for C4H is induced in ON buds. However, as opposed to the microarray results, one gene, *FLS*, showed no significant induction in the nCounter analysis. Other genes of the flavonoid biosynthesis pathway, phenylalanine ammonia-lyase (*PAL*), dihydroflavonol 4-reductase (*DFR*), flavanone 3-hydroxylase (*F3H*) and Anthocyanidin synthase (*AS*), showed no change in their transcript levels between ON and OFF buds. The expressions of all 11 genes were analyzed from May until January, but most of them showed no significant change between ON and OFF buds at the rest of the time points (Figure S5).

The flavonoid biosynthetic pathway was further investigated by metabolomic analysis of a few flavonoids in ON and OFF buds during May using UPLC-QTOF-MS. The following compounds were identified by accurate mass, fragmentation pattern and a few standards: naringin/narirutin, hesperidin/neohesperidin, poncirin/didymin (flavonones), diosmin (flavone). In agreement with the gene-expression analyses, the intensities of all tested com-

Table 1. Gene ontology (GO) categorization of genes induced in OFF buds.

GO term	Description	% in input list	% in BG/Ref	p-value	FDR	Fold enrichment
GO:0019748	secondary metabolic process	5.5336	1.2429	7.00E-10	6.60E-07	4.45
GO:0009698	phenylpropanoid metabolic process	3.1621	0.5005	2.20E-08	6.70E-06	6.32
GO:0009812	flavonoid metabolic process	2.5692	0.3149	2.00E-08	6.70E-06	8.16
GO:0009699	phenylpropanoid biosynthetic process	2.5692	0.4707	2.20E-06	0.00052	5.46
GO:0009813	flavonoid biosynthetic process	1.9763	0.3016	6.30E-06	0.0011	6.55
GO:0010017	red or far-red light signaling pathway	1.1858	0.0895	7.30E-06	0.0011	13.25
GO:0009639	response to red or far-red light	1.7787	0.2519	9.70E-06	0.0013	7.06
GO:0009821	alkaloid biosynthetic process	1.1858	0.1061	2.10E-05	0.0024	11.18
GO:0009820	alkaloid metabolic process	1.9763	0.3546	2.60E-05	0.0027	5.57
GO:0019438	aromatic compound biosynthetic process	2.9644	0.8120	5.10E-05	0.0048	3.65
GO:0009585	red, far-red light phototransduction	0.9881	0.0795	6.00E-05	0.0051	12.42
GO:0009583	detection of light stimulus	0.9881	0.0862	9.00E-05	0.0065	11.47
GO:0007602	phototransduction	0.9881	0.0862	9.00E-05	0.0065	11.47
GO:0006725	cellular aromatic compound metabolic process	4.5455	1.7699	0.00017	0.011	2.57
GO:0009582	detection of abiotic stimulus	0.9881	0.0994	0.00018	0.011	9.94
GO:0009581	detection of external stimulus	0.9881	0.1027	0.00022	0.013	9.62
GO:0051716	cellular response to stimulus	3.3597	1.1435	0.00024	0.013	2.94
GO:0042398	cellular amino acid derivative biosynthetic process	2.7668	0.8584	0.00032	0.017	3.22
GO:0009809	lignin biosynthetic process	1.1858	0.1823	0.00046	0.022	6.50
GO:0009808	lignin metabolic process	1.1858	0.1823	0.00046	0.022	6.50
GO:0006575	cellular amino acid derivative metabolic process	3.3597	1.2495	0.00064	0.028	2.69
GO:0009628	response to abiotic stimulus	4.7431	2.0815	0.00067	0.028	2.28
GO:0009791	post-embryonic development	2.5692	0.8352	0.00078	0.03	3.08
GO:0009416	response to light stimulus	2.5692	0.8319	0.00075	0.03	3.09
GO:0051606	detection of stimulus	0.9881	0.1359	0.00082	0.031	7.27
GO:0015979	photosynthesis	1.7787	0.4574	0.00096	0.034	3.89
GO:0009314	response to radiation	2.5692	0.8651	0.0011	0.037	2.97
GO:0009805	coumarin biosynthetic process	0.9881	0.1525	0.0014	0.045	6.48
GO:0009804	coumarin metabolic process	0.9881	0.1525	0.0014	0.045	6.48

BG, background; Ref, reference; FDR, false discovery rate.
doi:10.1371/journal.pone.0046930.t001

pounds were higher in OFF buds than ON buds, although with varied significance (Figure 9), suggesting that flavonoid biosynthesis is induced in OFF buds, allowing an increase in these four flavonoid groups.

Discussion

Bud population and morphology in ON and OFF trees

The effect of year 1 yield on the return bloom of year 2 was as expected: heavy yield resulted in a lower number of flowers and higher number of vegetative buds, whereas the opposite was true following a light yield (Figure 1). Overall, buds collected during year 1 from OFF trees had a ca. 95% chance of flowering, as compared to buds collected from ON trees which had a ca. 30% chance of flowering. No effect of year 1 yield was detected on mixed-type shoots (inflorescences containing flowers and leaves at various ratios), only on generative buds (inflorescences carrying only flowers with no leaves). Indeed, while fruit load and flowering manipulations by various means, such as defruiting, GA treatment and fall girdling, are highly effective on generative inflorescences, their effect on mixed-type shoots is not always significant [3,5,15,16,51–53].

In general, bud morphology and anatomy did not change significantly from May to January. This is in agreement with Lord and Eckerd's [31] original finding of microscopic bud break only being detectable in as late as the end of December, and macroscopic bud break being detectable about 2 weeks after that. However, in May, soon after flowering and fruit set, OFF buds were already significantly longer than ON buds. In Pixie mandarin, fruit has been shown to inhibit vegetative shoot development by reducing both their number and the number of nodes which can bear floral and vegetative shoots the following spring [5]. A reduction in the number of nodes during the ON year might well explain the difference in bud length between ON and OFF trees.

Table 2. Gene ontology (GO) categorization of genes induced in ON buds.

GO term	Description	% in input list	% in BG/Ref	p-value	FDR	Fold enrichment
GO:0010252	auxin homeostasis	0.9804	0.0331	6.10E-07	0.0005	29.58
GO:0006073	cellular glucan metabolic process	3.1373	0.7292	7.80E-06	0.0011	4.30
GO:0009312	oligosaccharide biosynthetic process	1.1765	0.0829	6.50E-06	0.0011	14.20
GO:0044042	glucan metabolic process	3.1373	0.7292	7.80E-06	0.0011	4.30
GO:0005992	trehalose biosynthetic process	0.9804	0.0464	4.50E-06	0.0011	21.13
GO:0046351	disaccharide biosynthetic process	1.1765	0.0762	3.80E-06	0.0011	15.43
GO:0044264	cellular polysaccharide metabolic process	3.1373	0.7524	1.20E-05	0.0014	4.17
GO:0005976	polysaccharide metabolic process	3.5294	0.9380	1.40E-05	0.0014	3.76
GO:0005991	trehalose metabolic process	0.9804	0.0597	1.80E-05	0.0017	16.43
GO:0005984	disaccharide metabolic process	2.5490	0.5966	5.60E-05	0.0046	4.27
GO:0009311	oligosaccharide metabolic process	2.5490	0.6099	7.00E-05	0.0053	4.18
GO:0042221	response to chemical stimulus	6.8627	2.9598	0.00011	0.0079	2.32
GO:0016137	glycoside metabolic process	2.5490	0.6529	0.00014	0.0088	3.90
GO:0005985	sucrose metabolic process	2.3529	0.5734	0.00015	0.0091	4.10
GO:0005982	starch metabolic process	2.3529	0.6032	0.00025	0.014	3.90
GO:0044262	cellular carbohydrate metabolic process	4.3137	1.6075	0.00027	0.014	2.68
GO:0016138	glycoside biosynthetic process	1.1765	0.1624	0.00035	0.017	7.24
GO:0010035	response to inorganic substance	1.5686	0.3281	0.00068	0.031	4.78
GO:0010038	response to metal ion	1.3725	0.2585	0.00078	0.034	5.31
GO:0009733	response to auxin stimulus	1.9608	0.5204	0.001	0.042	3.77

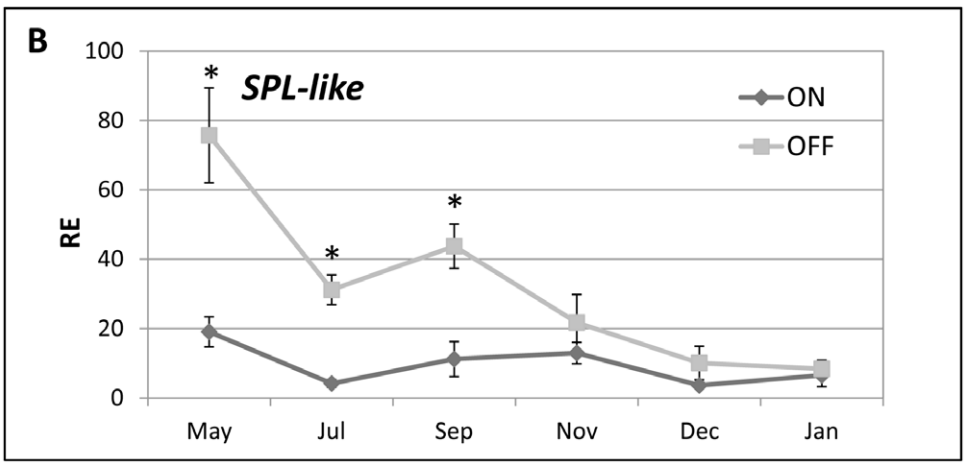
BG, background; Ref, reference; FDR, false discovery rate.
doi:10.1371/journal.pone.0046930.t002

Expression of flowering control genes in ON and OFF buds

To date, the expression of flowering control genes has been mostly investigated in leaves and stems. To the best of our knowledge, this work provides the only report describing the expression of flowering control genes in citrus buds. We recently demonstrated that during the flowering induction period, the mRNA levels of *FT* were considerably higher in buds than in leaves (Goldberg-Moeller R, Shalom L, Shlizerman L, Samuels S, Zur N, Ophir R, Blumwald E, Sadka A, Submitted). The mRNA levels of *FT2*, as well as of *AP1* and *LFY*, were higher in OFF buds than in ON buds during the flowering induction period, similar to that which has been described for *FT* and *AP1* in Moncada mandarin ON and OFF leaves [40]. Similarly, the expression of *FT*, *AP1* and *LFY* in mango was induced in leaves during the flowering induction period, with OFF trees showing higher levels of expression of *FT* and *AP1* [54]. Therefore, these results suggest that *FT2*, *LFY* and *AP1* might be involved in the annual phase transition in citrus. As the number of studied cases is so far too small, it is still difficult to generalize this picture to other perennial fruit trees [55]. Moreover, in apple, a deciduous tree, the expression of *AP1* and two *FT* genes was usually higher on fruit-bearing shoots than in succulent shoots [56]. The expression patterns of *CiFT1* and *CiFT3* provide similar picture to the two apple genes and a different one to *CiFT2*: first, ON buds displayed higher levels of their transcripts, and second, their induction did not occur during the flowering induction period, as with the other genes. In poplar, one of the *FT* paralogs' involvement in vegetative growth has recently been demonstrated [57]. It is therefore possible that *CiFT1* and *CiFT3* are involved in the control of vegetative rather than reproductive growth. There are three vegetative flushes in citrus: spring flush (February–March), summer flush (June–July) and fall flush (October–November). However, ON trees display suppressed vegetative growth. Therefore, we suggest that *CiFT1* and *CiFT3* either play a role in the suppression of vegetative flush development, or help determine ON bud fate toward vegetative growth the following spring. If the latter is indeed the case, the early induction of *CiFT1* and *CiFT3* should generate a signal that persists for a long time. If such a signal is indeed generated, then it should also be considered to be reversible, as the defruiting of ON trees induces flowering the following spring. Obviously, expression patterns provide only coincidental evidence for the involvement of the above genes in phase transitions. More direct evidence, such as that provided to establish the involvement of *FT*, *LFY* and *AP1* in the juvenile-to-adult phase transition, is required. However, one reasonable scenario (among others) is that *CiFT2*, *AP1* and *LFY* are induced in OFF buds and leaves in response to flowering-permissive environmental and endogenous signals. In ON organs, high fruit load prevents or reduces their induction by generating a 'negative AB signal'. The nature of the endogenous signal affected by fruit load, be it nutritional status of the tree, hormonal or some other signal(s), is currently unknown. However, while considering the various possibilities, the following points should be borne in mind: (1) like in *Arabidopsis*, it is reasonable to assume that more than one flowering control pathway exists in citrus [58,59], and therefore the effect of fruit load might be exerted on more than one pathway; (2) fruit load might also act via some exogenous signal, such as low temperature. As already noted, flowering in citrus is

A

	May	Sep	Dec-Jan
Bud FC (OFF/ON)	2.77	2.36	1.95
P Value	0.0002	0.0968	0.0630
LS FC (OFF/ON)	2.22	2.38	ND
P Value	0.0066789	0.0074575	



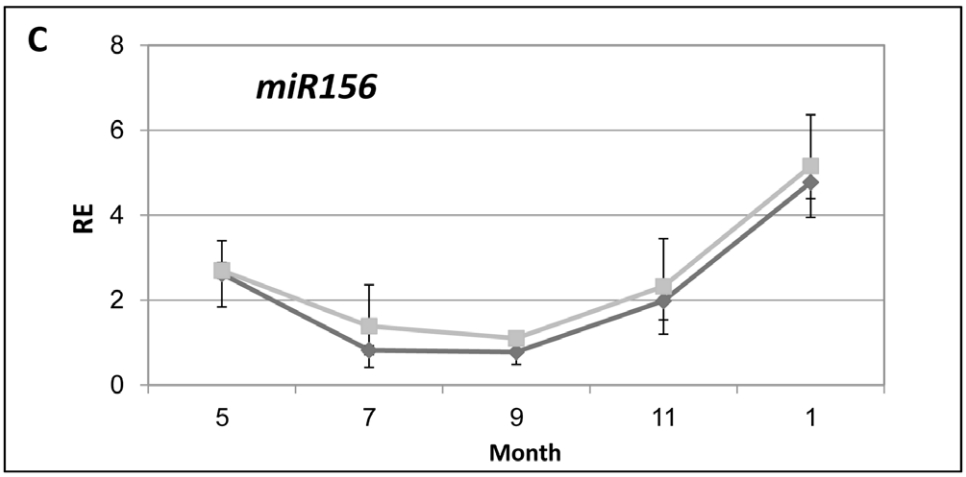


Figure 6. Expression of *SPL*-like and *miR156* in ON and OFF buds. Fold change (FC) between OFF and ON buds and leaves+stems (LS) of microarray probe Cit corresponding to *SPL*-like (A) in the indicated months. mRNA of *SPL*-like (B) and *miR156* (C) was analyzed in ON and OFF buds during the indicated months. Mean number of three biological replicates ± SE. Stars denote a significant difference between the expression of the gene in ON and OFF buds during the same time point ($P<0.05$).
doi:10.1371/journal.pone.0046930.g006

induced by low temperature, while day length has a relatively minor effect [30]; under permissive temperatures, shortening day length might induce more flowers, but has no effect under non-permissive temperatures. In *Arabidopsis*, the vernalization-flowering promotion pathway is dependent on the removal of *FLOWERING LOCUS C* (*FLC*) inhibition of *FT* expression in the leaves, and on the expression of *FD* and *SOC1* in the apical meristem [60]. However, to the best of our knowledge, *FLC*-like genes have not yet been described in plants other than crucifers, questioning the validity of the *Arabidopsis* mechanism in fruit trees. Moreover, although *SOC1* was induced during the flowering induction period (at least from September to December), no difference was detected between ON and OFF buds, in contrast to the case in leaves; (3) although day length has only a minor effect on flowering induction in citrus, day-length shortening, rather than temperature drop, might explain the 6-fold induction in *FT* expression from May to September in the OFF buds; (4) expression patterns are not always easily interpreted. For example, *AP1* expression was higher in ON than OFF buds in May, whereas that of *LFY* and *SOC1* was higher in OFF than ON buds. It might be that these genes have other functions at this stage.

One of the outcomes of the genomic analysis, validated by real-time PCR, was the induction in OFF buds and LS of the *SPL*-like gene (Figure 6). SPLs play a role in the juvenile-to-adult and annual phase transitions and are regulated by *miR156* [50]. Indeed, the importance of *miR156* in the juvenile-to-adult phase transition has been recently demonstrated in some trees [61]. In *Arabidopsis*, *SPLs* provide a gene family of 16 members, 10 of them regulated by *miR156* [62]. The citrus *SPL*-like seems to be a close relative of the small *SPL* genes, *SPL3/4/5*, based on three criteria: first, like *SPL3/4/5*, its *miR156*-binding site is located within the 3′UTR and not within the coding region as in other *SPL* family members (Shalom L, Shlizerman L, Blumwald E, Tumimbang E, Sadka A, in preparation); second, the putative SBP domain of

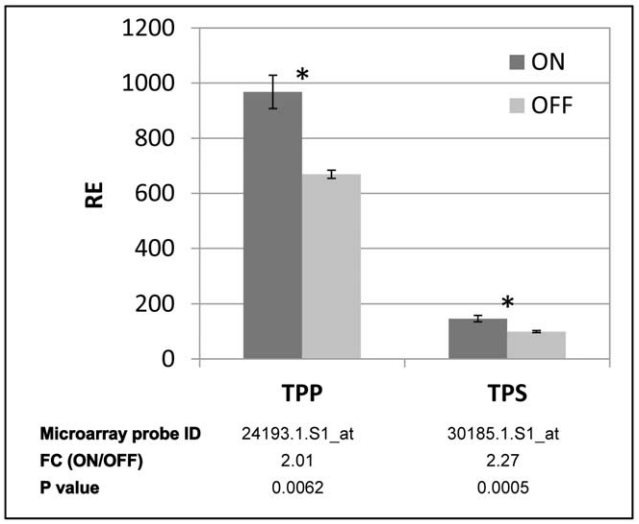


Figure 7. Expression of trehalose metabolism genes in ON and OFF buds. mRNA levels of trehalose phosphate phosphatase (TPP) and trehalose phosphate synthase (TPS) were measured in ON and OFF buds in May. Fold change (FC) between ON and OFF buds in their corresponding microarray probes are shown in the lower panel. Mean number of three biological replicates ± SE. Stars denote a significant difference in the expression of the gene between ON and OFF buds ($P<0.05$).
doi:10.1371/journal.pone.0046930.g007

citrus *SPL*-like shows highest homology to those of the *Arabidopsis SPL3/4/5*; third, similar to *SPL3/4/5*, its expected product is relatively small (130 amino acids). This gene's overall expression pattern in the buds suggests that it is negatively regulated by fruit load, and therefore might play a role in flowering induction following an OFF year. Functional analysis of the citrus gene in *Arabidopsis* showed that its overexpression induces early flowering, and that it possesses an active *miR156*-binding site (Shalom L, Shlizerman L, Blumwald E, Tumimbang E, Sadka A, in preparation). In *Arabidopsis*, SPLs act in both the leaf and the apical meristem to promote flowering in a complex manner involving several pathways [50]. In one of them, operated in the apical meristem, SPL3 and SPL9 bind to the promoter regions of flower meristem identity genes, inducing their expression. In this way, SPL promotes the expression of *FUL* and *LFY*, SPL9 promotes the expression of *SOC1* and *AGL42*, and both SPL3 and SPL9 promote the expression of *AP1*, in concert with the FT/FD complex [63]. The overall expression pattern of *SPL*-like in OFF buds compared to ON buds suggests that the gene responds to fruit load; the highest difference in mRNA levels, about 4-fold, was detected in May. This provides further support for the hypothesis that 'AB signal' is generated early in the season, at least 6 months prior to the flowering induction period. However, considering the action of SPLs in flowering induction, the overall reduction in this gene's mRNA levels is somewhat surprising. In fact, its mRNA levels are minimal during the flowering induction period, from November to January, when genes downstream of *SPL—LFY*, *SOC1*, *FT2* and *AP1*—are induced. It might be that SPL itself is regulated at the post-transcriptional level. Although less likely, it might be that in contrast to *Arabidopsis*, SPL does not act directly on the expression of flowering control genes, but generates a signal which acts during the flowering induction period. In any case, as already discussed, these results further emphasize the complexity in interpreting expression patterns. Further complexity stems from the pattern of expression of *miR156*: *miR256* was induced from September to January, in accordance with the reduction in *SPL* expression. However, its levels were slightly reduced from May to September, when *SPL* mRNA levels were also reduced. Moreover, no difference in its levels was detected between ON and OFF buds. These results suggest that *SPL* might be subjected to other modes of regulation, an option that is currently being investigated in our laboratory.

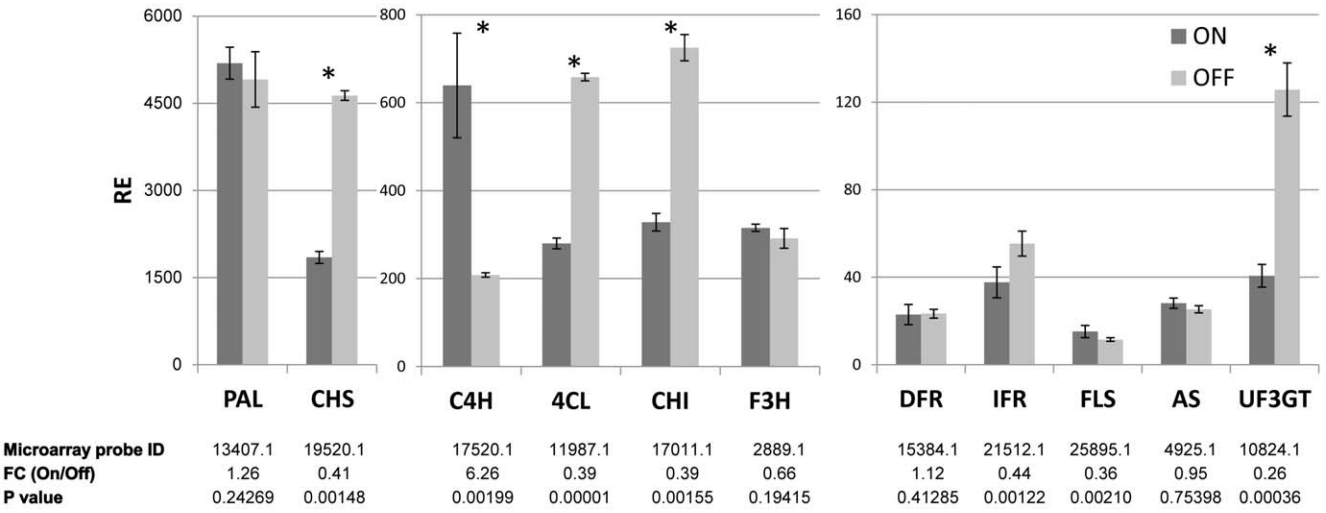


Figure 8. Expression of flavonoid biosynthetic pathway genes in ON and OFF buds. The mRNA levels of phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), cinnamate 4-hydroxylase (*C4H*), 4-coumarate:coenzyme A ligase (*4CL*), chalcone isomerase (CHI), flavanone 3-hydroxylase (*F3H*), dihydroflavonol 4-reductase (DFR), isoflavone reductase (*IFR*), flavonol synthase (FLS), Anthocyanidin synthase (*AS*) and UDP-glucose:flavonoid-3-O-glucosyltransferase (*UF3GT*) were measured in ON and OFF buds in May. Fold change (FC) between ON and OFF buds in their corresponding microarray probes are shown in the lower panel. Mean number of three biological replicates ± SE. Stars denote a significant difference in the expression of the gene between ON and OFF buds ($P<0.05$).
doi:10.1371/journal.pone.0046930.g008

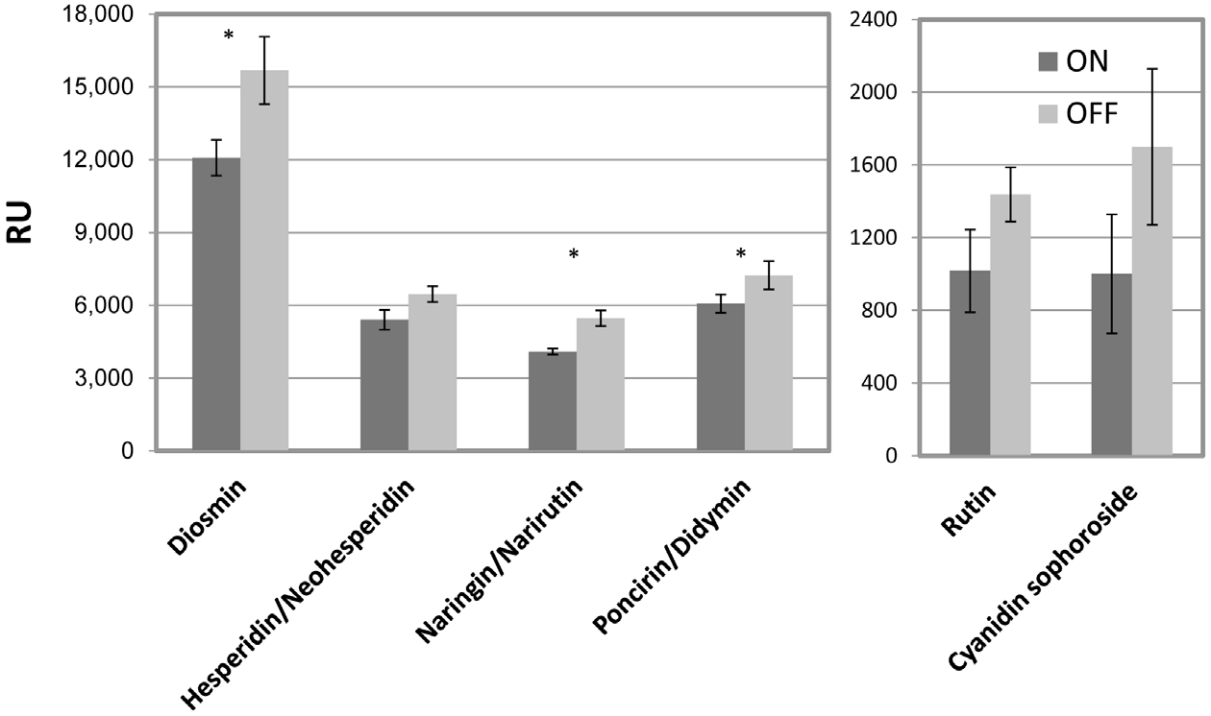


Figure 9. Flavonoid content in ON and OFF buds. The indicated flavonoids were measured in ON and OFF buds using LC-MS. Y axis indicates the intensity of each compound, RU relative units. Mean number of three biological replicates ± SE. Stars denote a significant difference between ON and OFF buds ($P<0.05$).
doi:10.1371/journal.pone.0046930.g009

Differentially expressed pathways in ON and OFF trees

The clustering analysis demonstrated that the impact of the three tested conditions, time, tissue type and AB state, on the level of similarity between the expression profiles follows the order: AB state>tissue type>time, i.e., ON and OFF organs showed more similar patterns than under the effect of time or tissue type (Figure 4A). This means that developmental changes over time within the same organ resulted in greater changes in gene expression than ON and OFF states of the organ at the same time point. However, it should also be noted that buds and LS showed more similar patterns at the same time point than buds or LS at different times. A similar gene expression pattern suggests that buds and LS share common functions, which is not surprising considering the fact that the bud contains leaf primordia.

Among the three analyzed time points, the largest number of DEPs between ON and OFF trees was detected in May, while in September and between November and January, their number was relatively low. Moreover, in May, the number of DEPs was much higher in buds than in LS. Taken together with the finding that the maximal difference in bud width develops between May and July, these results suggest that AB signal, if present, is generated much earlier than the flowering induction period, and causes the above changes. Alternatively, changes in gene expression in May might reflect changes in resource allocation: when fruit is present (ON trees), buds are deprived of photo-assimilates, which directly reduces their size in comparison to the case in OFF trees.

Biological processes affected by fruit load in May buds

As already noted, the highest number of DEPs was evident during May in the buds. Obviously, we cannot cover all of the metabolic processes which are induced at this time point, and we therefore briefly discuss three of these processes: two of them, flavonoid biosynthesis and photosynthesis, are induced in OFF buds, and one, trehalose metabolism, is induced in ON buds. The expression of genes belonging to two of these processes, flavonoid biosynthesis and trehalose metabolism, were also validated by nCounter technology.

Trehalose metabolism. ON buds showed increased expression of the two genes of trehalose metabolism, *TPS* and *TPP*. Trehalose is a disaccharide, which serves as an alternative sugar to sucrose in a variety of bacterial and fungal species [64]. In resurrection plants, where it serves as an osmoprotectant, trehalose is present at high levels, but usually in higher plants it is below detection levels. Changes in the trehalose biosynthetic genes and/or enzymes, and not necessarily trehalose levels themselves, were postulated to play a signaling or regulatory role in stress-response pathways [65]. Moreover, *Arabidopsis* plants mutated in *TPS* show arrested-growth phenotypes, remaining in the vegetative growth phases, suggesting that the gene is required for proper embryo development [66]. These results demonstrate the importance of the trehalose biosynthetic pathway for normal vegetative growth and transition to the flowering phase. An increase in the expression of TPS and TPP along with unchanged expression in trehalase, which catabolizes trehalose, suggests that trehalose level and/or pathway is induced in ON buds in May. These results can be explained in two ways. First, as a result of the high investment in developing fruits, ON trees are commonly under stress [7], which might directly affect the bud. Increased production of trehalose might play a role in mitigating the effects of these stresses. Second, the significant differences in *TPS* and *TPP* expression in buds, but not in LS, in May suggest a possible role for trehalose and/or its biosynthetic pathway in citrus flowering induction and in the regulation of AB itself, via an unknown mechanism.

Photosynthesis. Genes belonging to the three components of photosynthesis—light reactions, Calvin cycle and photorespiration—are induced in OFF buds (Figure S3, Table S6). In addition, SEA of expression showed that processes involved in the detection of light stimulus (including red/far red light) and phototransduction are also induced in OFF buds (Table 1). Bud morphology does not allow efficient photosynthesis and like the fruit, it provides a sink organ for photoassimilates. Moreover, while in ON trees, the bud competes with the developing fruit for resources, no such competition occurs in OFF trees, loaded with photoassimilates and storage molecules. In fact, according to nutritional theory, photoassimilate availability might well play a regulatory role in flowering induction. However, one could question the reason for inducing the photosynthetic machinery within the bud in an OFF year. We hypothesize that this induction provides a signal for the nutritional status of the bud. In other words, the bud signals its surrounding source leaves that it is loaded with photoassimilates, so the translocation rate is reduced. Although at this stage we cannot provide direct evidence for this hypothesis, it has been suggested that specific tissues within tomato fruit signal their sink strength by altering their photosynthetic machinery; indeed, different tissues possess different photoassimilate-translocation rates.

Flavonoid biosynthesis. Genes of a few pathways of secondary metabolism were induced in OFF buds, including flavonoids, phenylpropanoids, alkaloids and lignin (Table 1, Figure 5, Figure S6). Induction of five flavonoid biosynthesis genes was validated by nCounter technology, and metabolomic analysis confirmed that the pathway might indeed be induced in OFF buds in May (Figure 9, Figure S6). The induction of specific flavonoids was relatively marginal; however, the identification was limited by the standards used, and other flavonoids might also be induced. In any case, it seems that not only the central pathway was induced, but also the side reactions. Flavonoids are secondary metabolites that influence a variety of characteristics, such as aroma and flavor pigmentation, as well as protection against UV radiation [67]. Their synthesis has been hypothesized to occur under conditions of excess photoassimilates, particularly sucrose [68]; sucrose feeding of *Arabidopsis* plants has been shown to result in increased expression of flavonoid biosynthetic genes, especially those encoding anthocyanin [69]. In light of these findings, it is suggested that flavonoids in the bud serve as ''sink'' molecules for excess photoassimilates and other carbon molecules accumulating in the tree in OFF years.

In summary, results of this work show that a relatively long time before the flowering induction period, fruit load affects many regulatory and metabolic processes in the bud. Obviously, it should be considered that this and other conclusions of the work are based on a single cropping year. Although the expression of some of the flowering control genes was partially investigated during another year, with similar results (data not shown), year to year environmental and other external variations might affect the results, and therefore the conclusions. It should also be mentioned that the nature of the AB signal, and whether it is produced that early, remain open questions. Even if produced in May, or earlier, the signal must be reversible, as fruit thinning or complete removal from ON trees reverses the AB state. Ongoing studies in our laboratory include analyses of buds following fruit removal in September, when the number of differentially expressed genes between ON and OFF buds was very low. These analyses are expected to clarify which of the processes induced in ON and OFF buds are directly affected by fruit load. We are also investigating the possibility of *SPL*-like playing a role in AB signaling. In light of suggestions in the literature, the trehalose metabolism is involved in vegetative and reproductive growth, and the possible involvement of this metabolism in AB control warrants further study.

Supporting Information

Figure S1 The annual cycle in citrus. Stage I and Stage II of fruit development are as described previously [41].
(TIF)

Figure S2 ON and OFF shoots in citrus. Schematic description of OFF-year fruitless shoot and ON-year fruit bearing shoot. Buds are represented as brown triangle. Bud collection from OFF shoot was performed as described under Material and Methods. All buds of ON shoot were collected for the analyses.
(TIF)

Figure S3 Induction of photosynthesis in OFF buds. Differentially expressed probes were analyzed by MapMan. Blue squares represent genes induced in ON buds and red squares represent genes induced in OFF buds. A description of the specific genes and their fold change is provided in Table S6.
(TIF)

Figure S4 Expression of trehalose metabolism genes in ON and OFF buds. mRNA levels (RE) of trehalose phosphate phosphatase (TPP) and trehalose phosphate synthase (TPS) were measured in ON and OFF buds during the indicated months.
(TIF)

Figure S5 Expression of flavonoid biosynthetic pathway genes in ON and OFF buds. mRNA levels of phenylalanine ammonia-lyase (*PAL*), chalcone synthase (*CHS*), cinnamate 4-hydroxylase (*C4H*), 4-coumarate:coenzyme A ligase (*4CL*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), dihydroflavonol 4-reductase (*DFR*), isoflavone reductase (*IFR*), flavonol synthase (*FLS*), Anthocyanidin synthase (*AS*) and UDP-glucose:-flavonoid-3-O-glucosyltransferase (*UF3GT*) were measured in ON and OFF buds during the indicated months.
(TIF)

Figure S6 Induction of flavonoid pathway in OFF buds. A scheme showing the biosynthetic pathway of flavonoids. Genes induced in OFF buds in the microarray or in the real-time PCR are marked with squares. Standards for specific flavonoid groups are also marked.
(TIF)

Table S1 Primers list.
(XLSX)

Table S2 List of genes (http://www.phytozome.net/) used for nCounter analysis.
(XLSX)

Table S3 Microarrays hybridization results by log signal.
(XLSX)

Table S4 GO annotations for differentially expressed probes in May buds.
(XLSX)

Table S5 Probe list of Fig. 5.
(XLSX)

Table S6 Probe list of Fig. S3.
(XLSX)

Author Contributions

Conceived and designed the experiments: L. Shalom AS. Performed the experiments: L. Shalom SS NZ L. Shlizerman HZ MW. Analyzed the data:

L. Shalom RO AS. Contributed reagents/materials/analysis tools: EB. Wrote the paper: L. Shalom AS.

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