# Optimising citrus fruit size by regulating flower numbers and crop load

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CSIRO Plant Industry

Project Number: CT03031

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# HAL Ltd Project CT03031

# Final Report: 31 December 2005

# OPTIMISING CITRUS FRUIT SIZE BY REGULATING FLOWER NUMBERS AND CROP LOAD

**Steve Swain**

**CSIRO PLANT INDUSTRY**

**MERBEIN, VICTORIA**

HAL project number: CT03031 (31st December 2005)
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This is the final report for the project "Optimising citrus fruit size by regulating flower numbers and crop load" submitted 31st December 2005.

Funding sources for this project were obtained from CSIRO Food into Asia, a voluntary contribution of $120, 000 from the Murray Valley Citrus Board (MVCB) and matching funds from Horticulture Australia Ltd. This project was conducted in collaboration with the NSW Department of Primary Industries, Dareton Research Station.

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# MEDIA SUMMARY

Fruit size is a major issue for the Australian citrus industry. The tendency for citrus to exhibit a biennial bearing pattern of flower, and hence fruit, numbers has a significant impact on fruit size at harvest. In “on” years in which a large numbers of flowers are produced, an excessive number of fruit are set, often in clusters of several fruit on a single branch. Competition between the developing fruit for assimilates reduces the size of all fruit, unless chemical- or hand-thinning is used to reduce fruit numbers. Hand-thinning is labour-intensive and expensive while post-set chemical thinning can damage trees.

Previous research, both in Australia and overseas, demonstrates that one of the most effective ways of regulating crop load, and hence fruit size, is to regulate flower numbers with gibberellic acid (GA) sprays in winter. At present, a major difficulty with this approach is the inability to reliably predict flowering intensity in an orchard each spring, and hence the dosage of GA required in winter to achieve optimal flower and fruit numbers.

The overall aim of this project was to develop and test practical methods of predicting flowering intensity (a citrus floral index) in the coming spring by analysing dormant buds collected in winter for the activity of key flowering genes. This type of information would allow growers to use treatments such as winter gibberellic acid (GA) sprays more effectively to regulate flower production and hence fruit numbers and size.

Despite success in isolating citrus flowering genes and measuring their expression in dormant winter buds, a gene-based floral index test is not feasible at the present time. However, this project has successfully generated knowledge and molecular tools that could be used to develop DNA fingerprinting for identification of citrus varieties or to improve seedling selection in conventional citrus breeding.

# TECHNICAL SUMMARY

Fruit size is a major issue for the Australian citrus industry. The tendency for citrus to exhibit a biennial bearing pattern of flower, and hence fruit, numbers has a significant impact on fruit size at harvest. In "on" years in which a large numbers of flowers are produced, an excessive number of fruit are set, often in clusters of several fruit on a single branch. Competition between the developing fruit for assimilates reduces the size of all fruit, unless chemical- or hand-thinning is used to reduce fruit numbers. Hand-thinning is labour-intensive and expensive while post-set chemical thinning can damage trees.

Previous research, both in Australia and overseas, demonstrates that one of the most effective ways of regulating crop load, and hence fruit size, is to regulate flower numbers with gibberellic acid (GA) sprays in winter. At present, a major difficulty with this approach is the inability to reliably predict flowering intensity in an orchard each spring, and hence the dosage of GA required in winter to achieve optimal flower and fruit numbers.

The overall aim of this project was to develop and test practical methods of predicting flowering intensity (a citrus floral index) in the coming spring by analysing dormant buds collected in winter for the activity of key flowering genes. This type of information would allow growers to use treatments such as winter GA sprays more effectively to regulate flower production and hence fruit numbers and size. A prerequisite for such a predictive test is the identification of citrus flowering genes and the ability to measure their expression (i.e. activity) in dormant winter buds before possible GA treatments. This approach was chosen because simpler techniques used to predict flowering in other tree crops, such as microscopic analysis of buds or the "Merbein bunch count" for grapevines, cannot be applied to citrus prior to potential application of GA.

The function of selected citrus genes isolated in this project has been investigated by their ability to restore the floral or meristem identity defects in the corresponding Arabidopsis mutants. Based on these experiments *CsSL1* and *CsSL2* have a role in flower initiation and *CsWUS* is important for meristem development. The role of *CsAP3* has not been confirmed, and it is possible that this gene is a pseudogene that does not encode a functional protein. These physiological roles are consistent with the DNA sequence of the citrus genes and confirm that the genes controlling flowering and flower development are similar in all flowering plants. Significantly, the citrus *CsSL1* gene also affects flower organ maturation and abscission, suggesting that these genes could potentially be used to modify important traits such as fruit retention/abscission and maturation/senescence.

These results confirm that knowledge gained from genetic and molecular biology research in model plants such as *Arabidopsis* can facilitate the study of commercially important species such as citrus.

To allow comparison of the observed gene expression with flowering observed in the following spring, dormant winter buds and subsequent flowering data were obtained from the Ralex trial being conducted by Tahir Khurshid at Dareton, NSW. Buds from two years, an "on" year in 2003 and an "off" year in 2004, were analysed in detail. In bud samples from both years, no clear differences in the expression of flowering genes due to Ralex (200 ml/100 L) treatment were observed. This was despite the fact that Ralex was clearly effective at reducing flower numbers in 2003. A moderate change in *CsWUS* expression due to Ralex was observed in 2003, possibly suggesting that a floral index could be based on the expression of this gene. Unfortunately, *CsWUS* expression could not be detected in buds harvested in May, from trees with a range of crop loads, probably because of limitations in existing detection methodologies and the extremely small size at this early stage of the part of the bud from which flowers arise. Finally, when individual buds were examined from control trees in 2003, relatively little variation in the expression of a flowering gene (*CsAP1*) or in *CsWUS* was observed, suggesting that despite the range of shoot tissues produced in the

following spring, differences in gene expression between buds are limited. This result is consistent with the expression profiles of the genes tested in pooled bud samples in which expression of several flowering genes was similar with or without Ralex treatment.

The results described above suggest that, despite success in isolating citrus flowering genes and measuring their expression in dormant winter buds, a gene-based floral index test is not feasible at the present time. However, this project has successfully generated knowledge and molecular tools that could be used to develop DNA fingerprinting for identification of citrus varieties or to improve seedling selection in conventional citrus breeding.

# INTRODUCTION

The Australian Citrus industry spans all states (except Tasmania) with major production focused in the Riverland, Sunraysia, Murrumbidgee Irrigation Area (MIA) and Central Burnett/Emerald areas. Collectively the industry is worth in excess of $400 million (00/01). While the majority of Australian citrus is consumed by the domestic market, Australia began exporting citrus in 1981 and since then the value of exports has grown steadily. Currently the Australian export citrus industry is worth $190 million (00/01) making it the largest exporter of fresh fruit in the horticultural industries. Navel oranges make up the largest component of the export citrus value. The major Asian export markets for oranges are Hong Kong, Malaysia, Singapore and Japan which together with a number of other minor Asian markets are collectively valued at $145 million (00/01). The citrus industry anticipates that production will increase by 1.25-fold between now and 2010 with the bulk of new production expected to be absorbed by the Asian export markets. For example, China has recently been ratified as a member of the WTO, which will open up the Chinese and Taiwanese markets potentially leading to a 10-fold increase in market volume once appropriate export protocols can be developed.

Australia is a small producer of citrus, comprising less than 1% of global citrus production. In effect, our export markets are niche markets, sustained by 'out of season' supply and superior quality. The export industry is highly dependent on a small number of markets with, for example, the top five navel markets (four of which are in Asia) representing 87% of total export volume. The industry strategy is to further develop a number of key export markets such as China. These export markets are exposed to pressure from alternative suppliers such as South Africa, Uruguay and Argentina with increased competition from these competitors as well as from Egypt, California (USA), Japan, South Korea, Italy and Malaysia expected over the next 10 years.

(Note the information presented in the Industry Background section is sourced from the 2002 Australian Citrus Growers Association's 54$^{th}$ Annual Report; the Australian Citrus Growers Association web-site http://www.auscitrus.org.au/; and from key industry personnel)

Fruit size is a major issue for the industry. The tendency for citrus to exhibit a biennial bearing pattern of flower, and hence fruit, numbers has a significant impact on fruit size at harvest. In "on" years in which a large numbers of flowers are produced, an excessive number of fruit are set, often in clusters of several fruit on a single branch. Competition between the developing fruit for assimilates reduces the size of all fruit, unless chemical- or hand-thinning is used to reduce fruit numbers. Hand-thinning is labour-intensive and expensive while post-set chemical thinning can damage trees. In both cases the final size of the remaining fruit is still less than would have been achieved if fewer flowers had originally been present. The heavy crop tends to reduce the numbers of flowers produced in the following "off" year. If trees are not managed correctly, and often despite management, this pattern of "on" and "off" years repeats itself leading to alternating seasons of firstly too few fruit followed by seasons with too many undersized fruit. A further factor is the influence of environment, particularly winter temperatures, which also modifies flower production. Because of this environmental effect trees of a particular variety within a geographical area tend to be synchronised for their biennial pattern. While this behaviour simplifies management to some extent, it greatly exacerbates the overproduction of small fruit in "on" years. This can be such a problem that in 2002 the Murray Valley Citrus Board (MVCB) ran television commercials urging growers not to harvest undersized fruit for which markets may be impossible to find.

Many citrus varieties exhibit this pattern of biennial bearing. Previous research, both in Australia and overseas, demonstrates that one of the most effective ways of regulating crop load, and hence fruit size, is to regulate flower numbers with GA sprays in winter. At present, a major difficulty with this approach is the inability to reliably predict flowering intensity in

an orchard each spring, and hence the dosage of GA required in winter to achieve optimal flower and fruit numbers.

**AIM**

The overall aim of this project is to develop and test practical methods of predicting flowering intensity (floral index) in the coming spring by analysing dormant buds collected in winter for the activity of key flowering genes. This information would allow growers to use treatments such as winter gibberellic acid (GA) sprays more effectively to regulate flower production and hence fruit numbers and size.

**Identification of key citrus flowering genes**

- Use publicly available information from other plants, particularly Arabidopsis and perennial crops such as grapes and apple, to identify parts of putative citrus genes involved in flowering
- Conduct preliminary characterisation of these genes

**Expression of key citrus flowering genes in dormant buds**

- Develop methodologies to measure gene activity in dormant citrus flower buds harvested in winter
- Measure the activity of citrus flowering genes in buds harvested at various times in winter and spring to further define genes best suited to the prediction of flowering
- Conduct detailed characterisation of these genes using standard molecular-genetic approaches

**Predicting citrus flowering behaviour**

- Compare the observed activity of citrus flowering genes in winter with the observed flowering behaviour in spring on trees with various crop loads and with or without GA treatments
- Develop a floral index by comparing the range of expression observed for key flowering genes with the extremes of flowering behaviour observed in orchards

## Materials & Methods

The identification of candidate citrus flowering genes was accomplished using a range of standard molecular biology techniques including degenerate PCR, cloning and sequencing. Portions of candidate genes were compared with the DNA sequence of known flowering genes from other species, and full-length clones isolated for the most promising candidates. As direct analysis of gene function is not possible in citrus, candidate citrus flowering genes were tested for function in Arabidopsis, making use of plants lacking the putative equivalent Arabidopsis gene. Standard molecular-genetic techniques were used for this analysis in OGTR-approved PC2 facilities.

For analysis of gene expression in citrus, dormant winter buds, flowers and vegetative tissues were harvested from selected trees at appropriate developmental stages/chronological ages and stored at –80$^{o}$C until required. Ralex treatments were conducted by Tahir Khurshid, NSW Department of Primary Industries, Dareton.

The analysis of gene expression involves quantifying the amount of mRNA transcribed from the gene of interest. Initially, a protocol previously developed by CSIRO for mRNA extraction from mango was modified for use with citrus (see below), and good quality mRNA successfully isolated. For subsequent mRNA isolations, a Qiagen RNeasy kit was used.

**RNA extraction protocol from citrus buds**

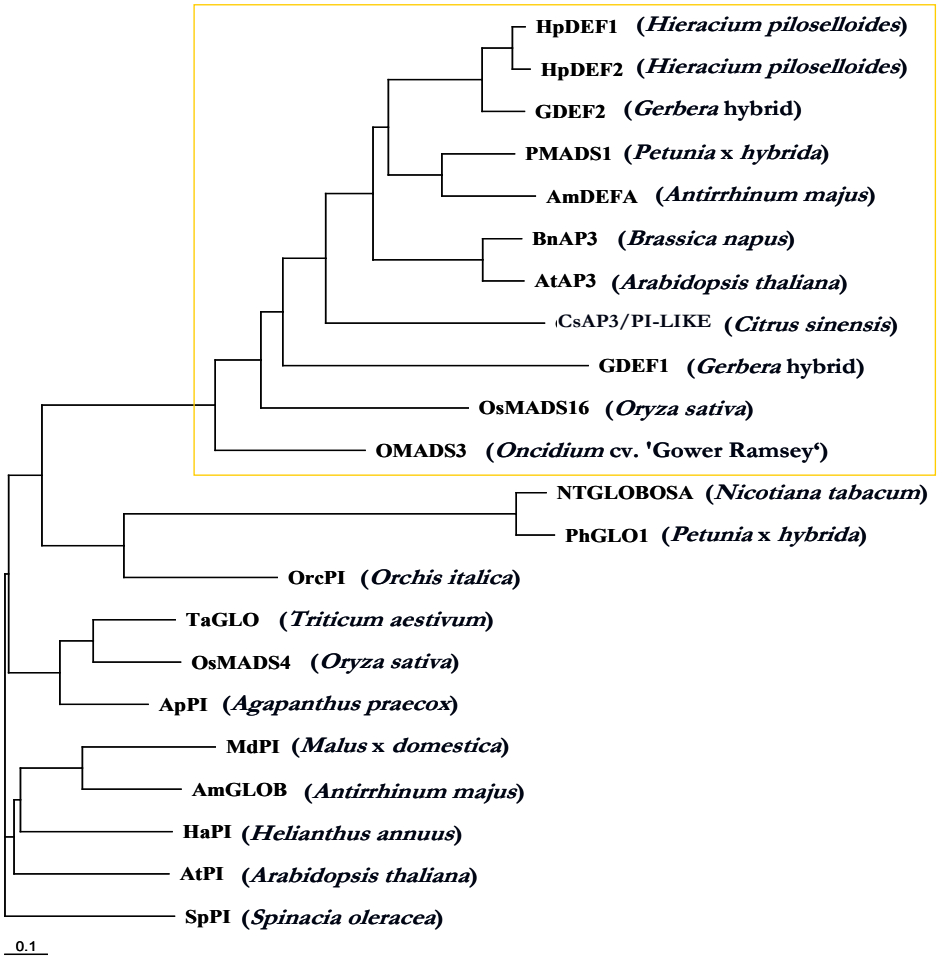
1. Approximately 0.1- 0.2 g of buds were ground in liquid $N_2$ using a pestle and mortar.
2. The powdery sample was then transferred to a 50 ml tube and 2.5 ml extraction buffer were added. The mixture was vortexed vigorously for 5 min.
3. The mixture was transferred to 1.5 ml eppendorf tubes in 500 µl aliquots.
4. The following components were added to each tube:
   55 µl 5M KoAc
   125 µl 100% EtOH
   and then vortexed vigorously for 3 min
5. An equal volume of chloroform:isoamylalcohol (24:1) was added and the sample was vortexed for another 3 min
6. The sample was then centrifuged at ~18,300 x g (15,300 rpm) for 10 min at 4$^{o}$C.
7. The top aqueous layer was recovered and then extracted twice with an equal volume of choloroform:isoamylalcohol (24:1). Following each extraction, the mixture was centrifuged at 18,300 x g for 10 min at 4$^{o}$C.
8. The recovered aqueous layer from the final extraction was pooled into 4 tubes. An equal volume of 6M LiCl was added to each tube.
9. The tubes were inverted several times before being left in a -20$^{o}$C freezer overnight.
10. The precipitated RNA was centrifuged at 18,300 x g for 30 min at 4$^{o}$C.
11. The pellet was washed twice with 300 µl 3M LiCl and then twice with 500 µl 80% EtOH. After every wash, the pellet was collected by centrifugation at 18,300 x g for 10 min at 4$^{o}$C.
12. The pellet was air-dried for 10-15 min and then dissolved in 300 µl TE buffer.
13. The following components were added: 18 µl 5M KoAc and 600 µl 100% EtOH.
14. The mixture was incubated at -80$^{o}$C for 30 min.
15. The precipitated RNA was collected by centrifugation at 18,300 x g for 30 min at 4$^{o}$C.
16. The pellet was washed twice with 500 µl 80% EtOH. After every wash, the pellet was collected by centrifugation at 18,300 x g for 10 min at 4$^{o}$C.
17. The pellet was then air-dried and resuspended in 25 – 30 µl DEPC-treated water.
18. The purified RNA was used immediately for first strand cDNA synthesis. Any excess RNA was stored at -80$^{o}$C.

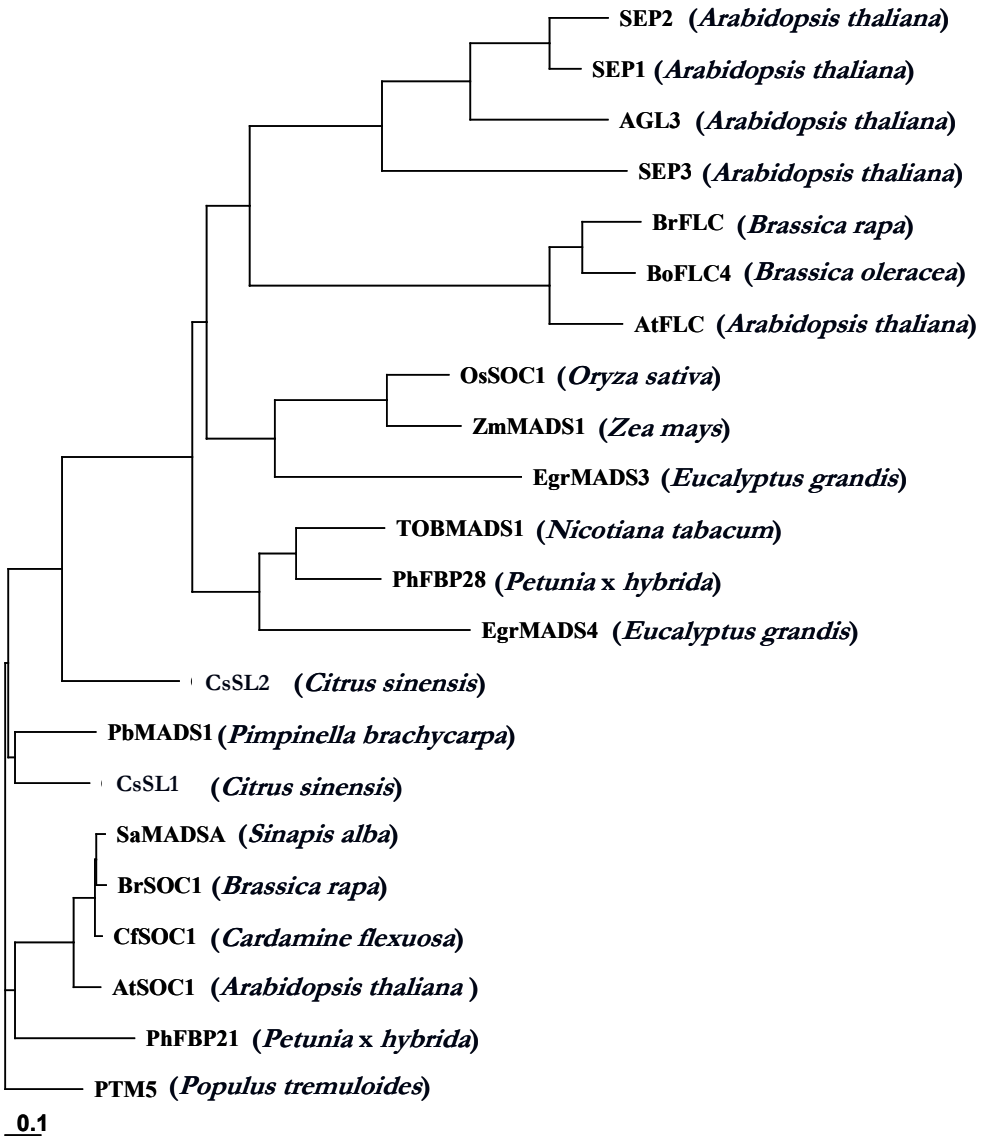
# RESULTS

## Identification of citrus flowering genes

Successful development and use of a citrus floral index requires the identification of citrus flowering genes. Based on the sequence of related genes from several plant species, degenerate PCR primers were designed to amplify candidate flowering genes from citrus. Once identified, a combination of 5' and 3' RACE was used to isolate full-length clones of selected genes. Three candidate flowering genes were identified, named *CsAPETALA3* (*CsAP3*), *CsSOC1-like1* (*CsSL1*) and *CsSOC1-like2* (*CsSL2*). Based on the predicted amino acid sequences, and consistent with the primers used, all are predicted to encode MADS-box proteins, a class of transcription factors known to play essential roles in flower initiation and development in a wide range of Angiosperm species (Figures 1,2).

**Figure 1.** Phylogenic trees indicating the relationship between proteins encoded by candidate citrus flowering genes (*CsAP3*, *CsSL1* and *CsSL2*) and similar genes from other species.



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sample, which is mostly composed of tissue types (ii) and (iii). Because the flowering genes act in the meristem cells, it is important to consider the expression of flowering genes relative to the amount of the bud meristem tissue. The most effective method of assessing meristem size in a gene-based test for flowering is to use a gene that is only expressed in meristem cells. Based on research conducted on other plants, we selected the *WUSCHEL* (*WUS*) gene for this purpose. As for the MADS-box genes, degenerate PCR was used to isolate a putative citrus version of this gene, named *CsWUSCHEL* (*CsWUS*) (Figure 2).

**Figure 2.** Predicted amino acid sequence comparisons. Citrus sequences are highlighted in grey.

**(i)** Sequence alignments of predicted proteins from 2 candidate citrus flowering genes (*CsSL1/2*) with similar proteins encoded by related genes from other plants.

```
EgrMADS4    MVRGKIQLRRIENTTSRQVTFSKRRNGLLKKAYELSVLCDAEVAVIIFSQKGRLYEFSSN 60
EgrMADS3    MARGKTKMRRIENATSRQVTFSKRRKGLLKKAYELSVLCEAEVAVIIFSQNGKLYEFSSN 60
CsSL2       MVRGKIQMKKIENDTSRQVTFSKRRNGMLKKAYELSVLCDAEVAVIIFSQKGRLYEFSS- 59
CsSL1       MVRGKTQMRRIENATSRQVTFSKRRNGLLKKAFELSVLCDAEVAVIIFSPRGKLSEFAS- 59
PTM5        MVRGKTQMRRIENATSRQVTFSKRRNGLLKKAFELSVLCDAEVALIVFSPRGKLYEFAS- 59
AtSOC1      MVRGKTQMKRIENATSRQVTFSKRRNGLLKKAFELSVLCDAEVSLIIFSPKGKLYEFAS- 59
CfSOC1      MVRGKTQMKRIENATSRQVTFSKRRNGLLKKAFELSVLCDAEVSLIIFSPKGKLYEFAS- 59
OsSOC1      MVRGKTQMKRIENPTSRQVTFSKRRNGLLKKAFELSVLCDAEVALIVFSPRGKLYEFAS- 59
ZmMADS1     MVRGKTQMKRIENPTSRQVTFSKRRNGLLKKAFELSVLCDAEVALVVFSPRGKLYEFAS- 59
            *.*** :::::** ***********:*:****:******:***::::** .*:* **:*

EgrMADS4    SEIQKTIDRYRRSTYDMDTYKTNLDQCILHLKQETTDMERKIELLEVSLRKLSGECLGSC 120
EgrMADS3    SEIRKTIDRYRRST-NVDTYQL-CGRYILHLKQETMDMERKIELLEVSQQKLSGQCLGSC 118
CsSL2       SEMQKTLERYYRYTEERQIDRNGMERYMQQLKHEIANMIEKIEHIEVSQRKLLGQDLGSR 119
CsSL1       SSMQETIERYLKHTKDTRNKQQPTEQNMQHLKHEAANMVKKIELLEVSKRKLLGEGLASC 119
PTM5        SSMQETIERYRRHVKENNTNKQPVEQNMLQLKEEAASMIKKIEHLEVSKRKLLGECLGSC 119
AtSOC1      SNMQDTIDRYLRHTKDRVSTKPVSEENMQHLKYEAANMMKKIEQLEASKRKLLGEGIGTC 119
CfSOC1      SNMQDTIDRYLRHTKDRVSSKPVSEENMQYLKFEAANMMKKIEQLEASKRKLLGEGIGTC 119
OsSOC1      ASTQKTIERYRTYTKENIGNKT-VQQDIEQVKADADGLAKKLEALETYKRKLLGEKLDEC 118
ZmMADS1     GSAQKTIERYRTYTKDNVSNKT-VQQDIERVKADADGLSKRLEALEAYKRKLLGERLEDC 118
            .. :.*::**   . :    :    . :  :* :  .: .::* :*.  :** *: :

EgrMADS4    SIDEIQMIGDQLERSLSSIRARKAQLFDDQIQHLQAKERSLKEENAKLLAKCLANPGQ-- 178
EgrMADS3    SINEIQEIGDQLEQSLSSIRKRKAQLFNDQIQQLQAKERHLKEENAKLLAKFLANPWQ-- 176
CsSL2       TNEELQELDDQLERSLRSIRARKAQLFNEQMGQLKEKERLLLEDNARLCIKCGQKPWQ-- 177
CsSL1       TLEELQQIERQLEKSVSNIRARKNQVFNEQIAQLKEKGKVLEAENTRLEEKCGMENWQ-- 177
PTM5        TIEELQQIEQQLERSVSTIRARKNQVFKEQIELLKQKEKLLAAENARLSDECGAQSWP-- 177
AtSOC1      SIEELQQIEQQLEKSVKCIRARKTQVFKEQIEQLKQKEKALAAENEKLSEKWGSHESE-- 177
CfSOC1      SIEELQQIEQQLEKSVKCIRARKTQVFKEQIEQLKQKEKALAAENGKLSEKWGSHETE-- 177
OsSOC1      SIEELHSLEVKLERSLISIRGRKTKLLEEQVAKLREKEMKLRKDNEELREKCKNQPPL-- 176
ZmMADS1     SIEELHSLEVKLEKSLHCIRGRKTELLEEQVRKLKQKEMSLRKSNEDLREKCKKQPPVPM 178
            : :*:: :  :**:*:  ** ** :::.:*:  *: *   *  .*  *  :   .

EgrMADS4    ----STAHPRAAALH---SRSSRSTDVETGLFIGLPELN---------------- 210
EgrMADS3    ----STAHPRAAAIN---SRSSRGTDVETGLFIGLPES----------------- 207
CsSL2       ----QSTQRKEAVNNC--SQSGQSSDIETELFIGLPEMRAA-------------- 212
CsSL1       ----GSKEQPENLTND---DGASTSDVETELFIGPPPERRARRLAIPPQN----- 220
PTM5        ----VSWEQRDDLPREEQRESSSISDVETELFIGPPETRTKR---IPPRN----- 220
AtSOC1      ----VWSNKNQESTGRGDEESSPSSEVETQLFIGLPCSSRK-------------- 214
CfSOC1      ----VWSNKNQES-GRGDEESSPSSEVETQLFIGLPCSSRK-------------- 213
OsSOC1      -SAPLTVRAEDENPDRNINTTNDNMDVETELFIGLPGRSRSSGGAAEDSQAMPHS 230
ZmMADS1     ASAPPRAPAVDNVEDGHREPKDDGMDVETELYIGLPGRDYRSS-KDKAAVAVRSG 232
                                          ::** *:** *
```

OsSOC1 = *Oryza sativa*
ZmMADS1 = *Zea mays*
AtSOC1 = *Arabidopsis thaliana*
CsSL1 = *Citrus sinensis*
CsSL2 = *Citrus sinensis*
PTM5 = *Populus tremuloides*
CfSOC1 = *Cardamine flexuosa*
EgrMADS3 = *Eucalyptus grandis*
EgrMADS4 = *Eucalyptus grandis*

**(ii)** Sequence alignments of the predicted protein from a candidate citrus flowering gene (*CsAP3*) with similar proteins encoded by related genes from other plants.

```
AmGLOB   MGRGKIEIKRIENSSNRQVTYSKRRNGIMKKAKEISVLCDAHVSVIIFASSGKMHEFCSP 60
ApPI     MGRGKIEIKRIENSTNRQVTFSKRRNGIIKKAREISVLCESQVSVVIFSSCGKMSEYCSP 60
MdPI     MGRGKVEIKRIENSSNRQVTYSKRRNGIIKKAKEITVLCDAKVSLIIYSSSGKMVEYCSP 60
AtPI     MGRGKIEIKRIENANNRVVTFSKRRNGLVKKAKEITVLCDAKVALIIFASNGKMIDYCCP 60
AtAP3    MARGKIQIKRIENQTNRQVTYSKRRNGLFKKAHELTVLCDARVSIIMFSSSNKLHEYISP 60
AmDEFA   MARGKIQIKRIENQTNRQVTYSKRRNGLFKKAHELSVLCDAKVSIIMISSTQKLHEYISP 60
CsAP3    MGRGKMEMKRIENATNRQVTFSKRRNGLFKKARELTILCDAKVSILICSSTAKAHEYISP 60
OMADS3   MGRGKIEIKKIENPTSRQVTYSKRRLGITKKAMELTVLCDAKVSLIMFSSSGKLSDYCSP 60
         *.***:::*:*** ..* **:**** *: *** *:::**:::*:::: :*  *  :: .*

AmGLOB   STTLVDMLDHYHKLSGKRLWDPKHEHLDNEINRVKKENDSMQIELRHLKGEDITTLNYKE 120
ApPI     NTSFPRILERYQHNCGKKLWDAKHENLNAQIDRVKKENDNMQIELRHLKGEDLNSLNPKE 120
MdPI     STTLTEILDKYHGQSGKKLWDAKHENLSNEVDRVKKDNDSMQVELRHLKGEDITSLNHVE 120
AtPI     SMDLGAMLDQYQKLSGKKLWDAKHENLSNEIDRIKKENDSLQLELRHLKGEDIQSLNLKN 120
AtAP3    NTTTKEIVDLYQTISDVDVWATQYERMQETKRKLLETNRNLRTQIKQRLGECLDELDIQE 120
AmDEFA   TTATKQLFDQYQKAVGVDLWSSHYEKMQEHLKKLNEVNRNLRREIRQRMGESLNDLGYEQ 120
CsAP3    STTTKQLLDLYQKTLRVDLWSSHYEKMLENLGAVEQVNRILKKQIRQRMGESLNDLTLEE 120
OMADS3   STEIKDAFQRYQQVTGFDIWDAQYQRMQSTLMNLREVNHKLQMEIRQRKGENLEGLDVKE 120
         .      .: *:      :* .::::.      : : *  :: ::::  ** :  *   :

AmGLOB   LMVLEDALENGTSALKNKQMEFVRMMRKHNEMVEEENQSLQFKLRQMHLDPMNDNVMESQ 180
ApPI     LIPIEEALENGLNGVRAKQMEYLKMLKKNERLLEEENKRLTYILRHQQLA-MEGNVRQ-- 177
MdPI     LMALEEALENGLTSIRDKQSKFVDMMRDNGKALEDENKRLTYELQKQQEMKIKENVRN-- 178
AtPI     LMAVEHAIEHGLDKVRDHQMEILISKRRNEKMMAEEQRQLTFQLQQQEMA-IASNARG-- 177
AtAP3    LRRLEDEMENTFKLVRERKFKSLGNQIETTKKKNKSQQDIQKNLIH--ELELRAEDPHYG 178
AmDEFA   IVNLIEDMDNSLKLIRERKYKVISNQIDTSKKKVRNVEEIHRNLVL--EFDARREDPHFG 178
CsAP3    LTGLEQDILDGLKIIRECKDQVLARQINTFKRKVRGVQKENKSLQDGFMINAKEEDPHYE 180
OMADS3   LRGLEQKLEESIKIVRERKYHVIATQTDTYKKKLRSTREMYPALLN-ELQEVDDENQQRS 179
         :  : . : .    ::  : . :       .      .     *          :

AmGLOB   AVYDHHHHQNIA----DYEAQMPFAFRVQPMQPNLQERF----------------- 215
ApPI     --LDLGYHQRER----EFAAQMPMAFRVQPIHPNLQQNK----------------- 210
MdPI     --MENGYHQRQLGNYNNNQQQIPFAFRVQPIQPNLQERI----------------- 215
AtPI     --MMMRDH------------DGQFGYRVQPIQPNLQEKIMSLVID----------- 208
AtAP3    --LVDNGGDYDSVLGYQIEGSRAYALRFHQNHHHYYPNHGLHAPSASDIITFHLLE 232
AmDEFA   --LVDNEGDYNSVLGFPNGGPRIIALRLPTNHHP-----TLHSGGGSDLTTFALLE 227
CsAP3    YELVDNGEHCDSDFGFQNEGPGIFALRLQPN------------------------- 211
OMADS3   FIAEDLSGVYNSAISMANQR---LAHCL---------------------------- 204
                               .  .
```

AmGLOB = *Antirrhinum majus*
AmDEFA = *Antirrhinum majus*
ApPI = *Agapanthus praecox*
AtAP3 = *Arabidopsis thaliana*
AtPI = *Arabidopsis thaliana*
CsAP3 = *Citrus sinensis*
MdPI = *Malus domestica*
OMADS3 = Oncidium cv. 'Gower Ramsey'

**(iii)** Sequence alignments of the predicted protein from a candidate citrus flowering/meristem gene (*CsWUS*) with similar proteins encoded by related genes from other plants.

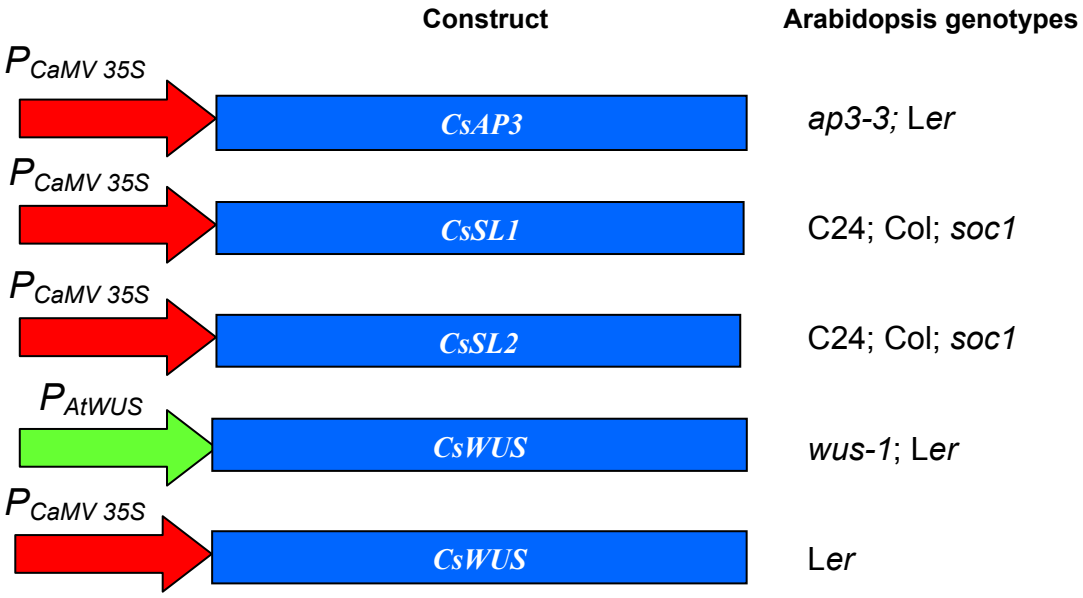
```
PhWUS       METAQHQQNNQQHYLHQHLSIGQGTNIEDGSNKNNSSNFMCRQNSTRWTPTTDQIRILKD 60
LeWUS       ---MEHQHN-----------------IEDGG-KNSNNSFLCRQSSSRWTPTSDQIRILKD 39
CsWUS       MEPQQQQNQHQ--------------GNGACGGSGKGNNCHCRPTCPRWTPTTDQIRILKE 46
ROA         MEPQQQQQQQGNEQQDSQ-------GIGKINNGSGGSSFLCRQSSTRWTPTTDQIRILKD 53
AtWUS       MEPPQHQHHHHQADQ----------ESGNNNNKSGSGGYTCRQTSTRWTPTTEQIKILKE 50
                ::*::                     .  . ...  ** ...*****::**:***:

PhWUS       LYYNNGVRSPTAEQIQRISAKLRQYGKIEGKNVFYWFQNHKARERQKKRLIAAATTDNTN 120
LeWUS       LYYNNGVRSPTAEQIQRISAKLRQYGKIEGKNVFYWFQNHKARERQKKRLIAAASATDNN 99
CsWUS       LYYNNGVRSPTAEQIQKISARLRQYGKIEGKNVFYWFQNYKARERLKKKIEGSSTSAADN 106
ROA         LYYNNGVRSPTAEQIQRISAKLRQYGKIEGKNVFYWFQNHKARERQKKRF-----TADHH 108
AtWUS       LYYNNAIRSPTADQIQKITARLRQFGKIEGKNVFYWFQNHKARERQKKRFNGTNMTTPSS 110
            *****.:*****:***:*:*:***:**************:***** **::     :

PhWUS       LPMQMQFQRGVWRSSADDPIHHKY-------TNPGVHCPSASSHGVLAVG--QNGNHGYG 171
LeWUS       NISSMQMIPHLWRSPDD---HHKYNTA---TTNPGVQCPSPSSHGVLPVV--QTGNYGYG 151
CsWUS       LPMHQRPAAATNWKPEDFANKSRSQS----ITSAGVSATLP-SYSVYTGG--QMGDHGYG 159
ROA         HHMNVPTIHNHHYKPPP--VYNKFSN----MNSGSFPSSSNGSPGFLTTPGSHVGNYGYG 162
AtWUS       SPNSVMMAANDHYHPLLHHHHGVPMQRPANSVNVKLNQDHHLYHHNKPYPSFNNGNLNHA 170
                        .                 .  .           .    : *: .:.

PhWUS       ALAMEKSFRDCS---ISP-GSSMSH-HHHQNFAWAGVDPYS-----STTTYPFLEKT--K 219
LeWUS       TLAMEKSFRECS---ISPPGGS-----YHQNLTWVGVDPYNNMSTTSPATYPFLEKSNNK 203
CsWUS       PVTMEKNFRDCS---ISSTGSSVVGGSRSQNYGWVGIDPHT-------SSYIFFGQKNSA 209
ROA         SVAMEKSFRECT---ISSTTDANVGGSMSQNIAWIGINNEY------HNPYTFIDTRKYM 213
AtWUS       SSGTECGVVNASNGYMSSHVYGSMEQDCSMNYNNVGGGWANMDHHYSSAPYNFFDRAKPL 230
            .   * .. :.:   :*.   .        *    * .           .* *:

PhWUS       HFENETLEADEEQQEEDQENYYYQRTTSAIETLPLFPMHEENISSFCNLKHQESSGGFYT 279
LeWUS       HYE-ETLD--EEQEEEN-----YQRGNSALETLSLFPMHEENIISNFCIKHHESSGG--- 252
CsWUS       DGN-QGNDKEDEEEEEN--------GHPGIETLPLFPMHGEDSINNYWNSKPNSSSYYSG 260
ROA         NGYDQTLEIEEEAEENY---------TAEIETLPLFPMHAD--------IKQDTADYFNG 256
AtWUS       FGLEGHQDEEECGGDAY---------LEHRRTLPLFPMHGEDHIN-------GGSGAIWK 274
                   :  :   :               .**.***** :             :.

PhWUS       EWYRADD-NLAAARASLELSLNSFIGNSS--- 307
LeWUS       -WYHSDN-NNLAA---LELTLNSFP------- 272
CsWUS       -WYGSNDGSSTSSSASLELSLNSYTSGSSGSI 291
ROA         ---RLENGCPRAS---LELTLNSWFGNSKYN- 281
AtWUS       --YGQSEVRPCAS---LELRLN---------- 291
                 .:    ::   *** **
```

AtWUS = *Arabidopsis thaliana*
CsWUS = *Citrus sinensis*
PhWUS = Petunia x hybrida
LeWUS = *Lycopersicon esculentum*
ROA = *Antirrhinum majus*

## Functional analysis of citrus flowering genes in Arabidopsis

Selected citrus candidate genes were analysed for their biological function by determining the ability of these genes to complement the developmental defects in the corresponding *Arabidopsis* floral or meristem identity mutants. To express the citrus genes in *Arabidopsis*, the protein coding sequence of each gene was first amplified from the respective cDNA sequences and then cloned into a plant expression vector under the direction of a CaMV 35S promoter. In addition, as ectopic *WUS* expression can be detrimental, the *Arabidopsis AtWUS* promoter was used to drive *CsWUS* expression (Figure 3). The expression cassettes were introduced into the *Arabidopsis* genome using the *Agrobacterium* mediated transformation technique.

**Figure 3.** Constructs for the transformation of Arabidopsis wildtype, flowering or meristem mutant plants. L*er*, Col and C24 are all wildtype ecotypes. *ap3-3* and *soc1* are flower development and flowering time mutants, respectively. *wus-1* is a meristem-defective mutant.




The Arabidopsis *SOC1* locus encodes a promoter of flowering, and mutant *soc1* plants exhibit late flowering due to a delay in the transition from the juvenile (non-flowering) to mature (flowering) stage under a range of environmental conditions. Ectopic *CsSL1* and *CsSL2* expression in the wildtype backgrounds L*er*, Col and C24 (Figure 4) caused an earlier transition to flowering suggesting that these citrus genes promote flowering. The over-expression of *CsSL1* and *CsSL2* in a *soc1* mutant also reduced the time to flowering (Table 1) demonstrating that these citrus genes can functionally substitute for the Arabidopsis gene. These results suggest that *CsSL1* and *CsSL2* are likely to have a similar function in citrus and may be involved in both the transition from juvenile (non-flowering) to mature (flowering) stage as well as production of new flowers each spring.

The 35S:SL1 and 35S:SL2 constructs also caused flower reiteration (i.e. secondary flowers initiated inside the carpels of some flowers), changed carpel (fruit) development and prolonged the life-span of floral organs (Figure 5).

**Figure 4.** Expression of the *CsSL1* and *CsSL2* transgenes in transformed Arabidopsis plants. The "–RT" panel represents a control experiment to confirm that the mRNA sample is not contaminated with DNA.

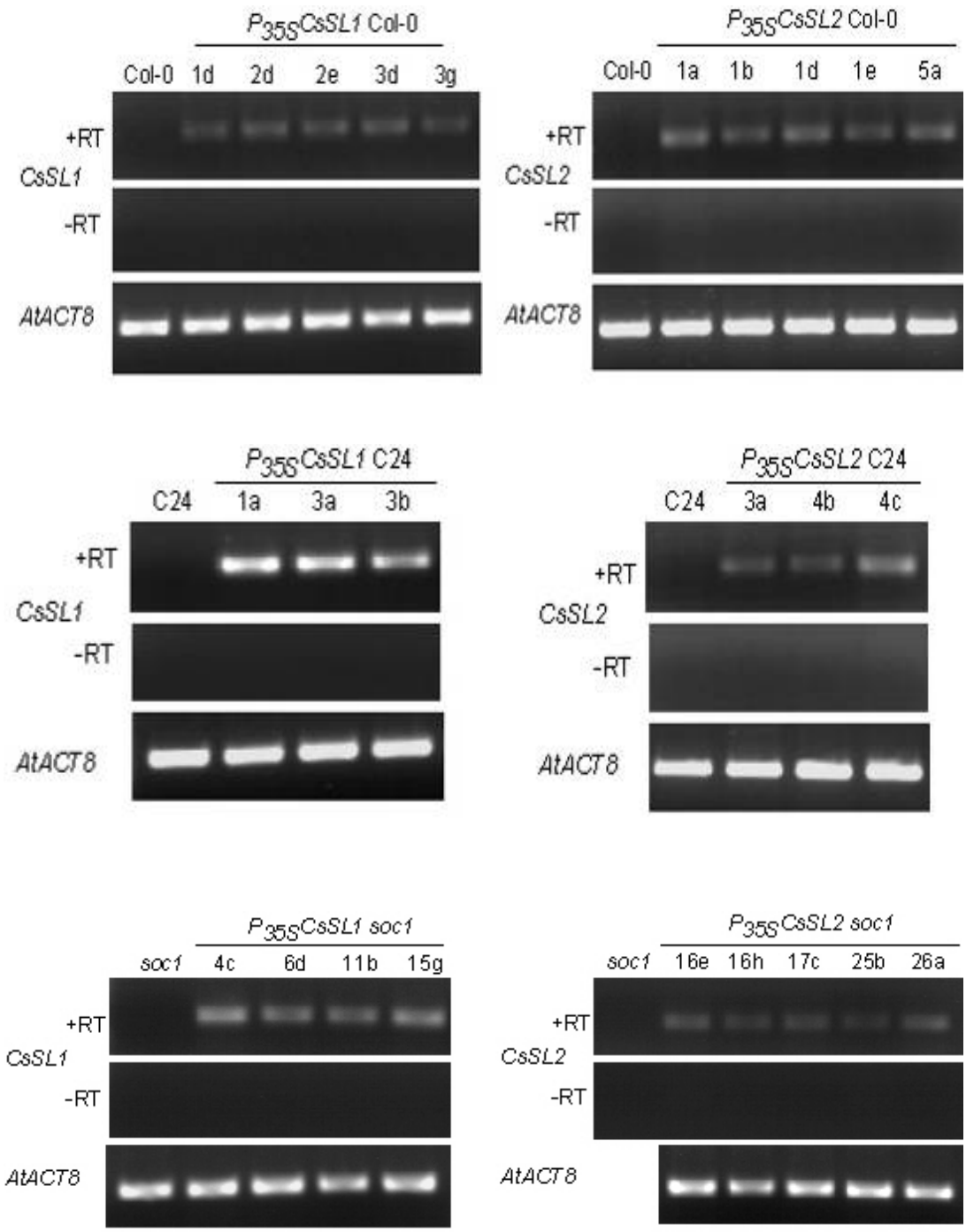


**Table 1.** Flowering time, as measured by the number of rosette leaves, in wildtypes L*er*, Col and C24, and *soc1* plants with or without the 35S:SL1 or 35S:SL2 constructs.

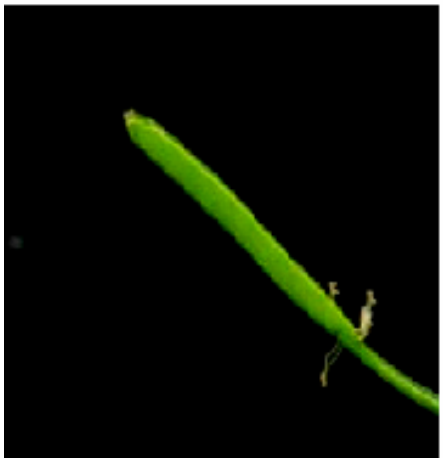
| Plant Genotype | Number of Plants | Number of Rosette Leaves +/-SD |
|---|---|---|
| L*er*-0 | 10 | 6.50±0.85 |
| Col-0 | 14 | 9.21±0.89 |
| C24 | 13 | 17.92±4.50 |
| *soc1/soc1* L*er*-0 | 15 | 9.93±1.03 |
| $P_{35S}$*CsSL1 soc1* L*er* | | |
| Line 4c | 4 | 3.25±1.50 |
| Line 6d | 6 | 3.00±1.10 |
| Line 11b | 15 | 7.13±1.64 |
| Line 15g | 15 | 3.93±1.28 |
| $P_{35S}$*CsSL2 soc1* | | |
| Line 16e | 15 | 5.93±0.88 |
| Line 16h | 15 | 5.27±0.59 |
| Line 17c | 15 | 8.80±1.78 |
| Line 25b | 15 | 6.13±0.83 |
| Line 26a | 15 | 7.13±1.06 |
| $P_{35S}$*CsSL1* Col-0 | | |
| Line 1d | 15 | 4.40±1.99 |
| Line 2d | 4 | 2.00±0.00 |
| Line 2e | 12 | 2.42±0.79 |
| Line 3d | 15 | 3.33±0.82 |
| Line 3g | 15 | 2.00±0.00 |
| $P_{35S}$*CsSL2* Col-0 | | |
| Line 1a | 15 | 6.93±0.96 |
| Line 1b | 15 | 8.53±1.19 |
| Line 1d | 15 | 5.67±1.05 |
| Line 1e | 15 | 5.87±0.52 |
| Line 5a | 15 | 5.53±0.64 |
| $P_{35S}$*CsSL1* C24 | | |
| Line 1a | 9 | 12.56±3.71 |
| Line 3a | 15 | 4.27±0.70 |
| Line 3b | 5 | 9.20±2.17 |
| $P_{35S}$*CsSL2* C24 | | |
| Line 3a | 15 | 13.40±2.64 |
| Line 4b | 14 | 14.71±5.54 |
| Line 4c | 13 | 21.54±5.25 |

**Figure 5.** The senescence of floral organs was delayed in wildtype and *soc1* Arabidopsis plants carrying the 35S:CsSL1 construct. This construct also alters carpel development and leads to occasional re-iteration of flowers within flowers. Fruit from *soc1* plants, which are indistinguishable from wildtype fruit, are shown as a control.

a) $P_{35S}$*CsSL1 soc1*

b) $P_{35S}$*CsSL1* Col-0

*soc1/soc1*



The Arabidopsis *AP3* locus encodes a MADS-box protein required for the correct formation of flower whorls 2 and 3: the petals and stamens. Mutant *ap3-3* plants produce sterile defective flowers that consist of sepals and carpels only. When *CsAP3* was constitutively expressed in plants carrying a strong *ap3-3* mutation, the over-expression of *CsAP3* was not sufficient to overcome the inability of the mutant plants to form proper petals and stamens. Thus, the function of the citrus *AP3-like* gene can not be confirmed from the existing data.

**Figure 6.** Constitutive expression of the citrus *AP3*-like gene was unable to rescue the floral organ defect of the *Arabidopsis ap3-3* mutant.

**L*er*** ***ap3-3 ap3-3*** ***ap3-3* + 35S:CsAP3**

Because ectopic *AtWUS* expression can cause severe developmental abnormalities, constructs were designed to drive expression of *CsWUS* from both the 35S and the Arabidopsis *AtWUS* promoters. Consistent with ectopic expression of *WUS* from other plants, only a single 35S:CsWUS transgenic line could be recovered. This line did not exhibit an obvious phenotype and was not examined further. A likely explanation for this result is that the majority of 35S:CsWUS transformants were seed or seedling lethal and the single line recovered contains a defective transgene with little or no *CsWUS* expression. Wildtype plants containing the AtWUS:CsWUS construct also failed to exhibit a detectable phenotype and were not examined further.

*CsWUS*, when expressed under the control of the *Arabidopsis AtWUS* promoter (Figure 7), was able to partially complement the meristem malfunction phenotype of the *wus-1* mutant (Figure 8). In AtWUS:CsWUS plants, the "bushy" appearance largely disappeared and was replaced with normal-looking shoot development; the inflorescence stems terminated in bunches of flowers, which in some transgenic lines possessed both stamens and carpels similar to wildtype flowers. Thus, *CsWUS* is capable of restoring almost all of the meristem functions in the *wus-1* mutant. This suggests that *CsWUS*, like its *Arabidopsis* counterpart, plays an important role in meristem development and is likely to be essential for shoot and flower formation in citrus.

**Figure 7.** Expression of the *CsWUS* transgene in transformed *wus-1* mutant plants. The "–RT" panel represents a control experiment to confirm that the mRNA sample is not contaminated with DNA.

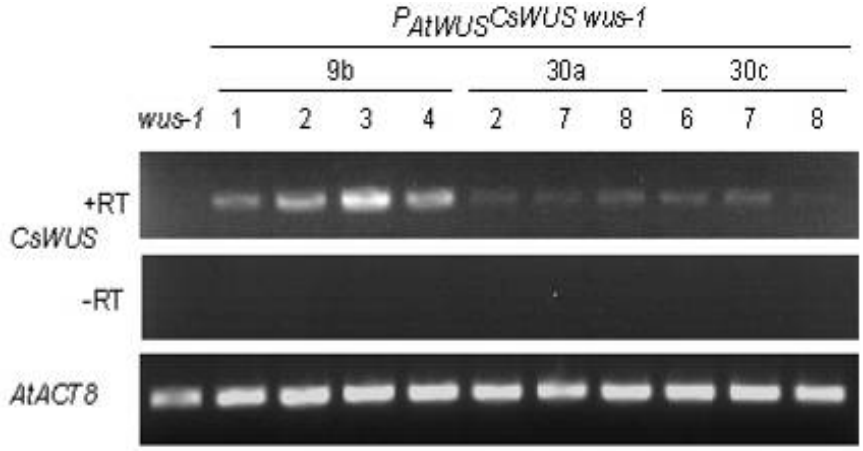
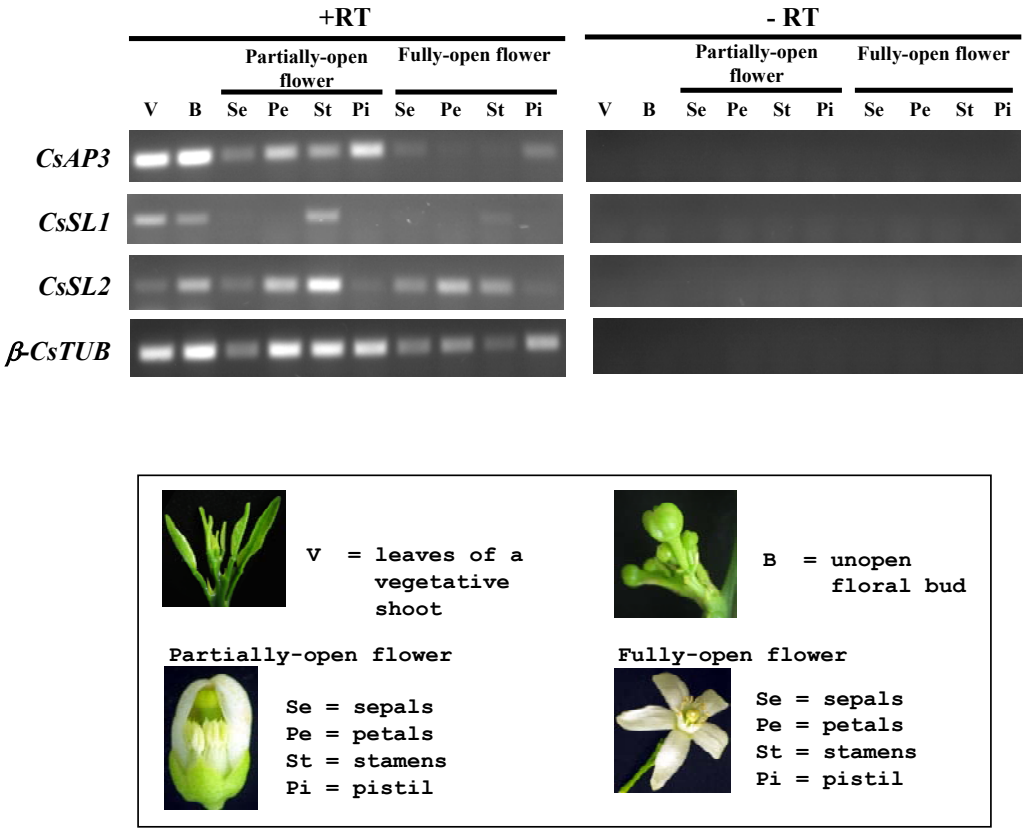



**Figure 8.** The Arabidopsis *wus-1* mutant phenotype was partially rescued when the citrus *CsWUS* gene was expressed under the control of the *Arabidopsis AtWUS* promoter.

## Analysis of gene expression in citrus

To further confirm the potential role of the selected citrus genes in flowering, expression was analyzed in different citrus organs (Figure 9). All three MADS-box genes, *CsAP3*, *CsSL1* and *CsSL2*, are expressed in various flower organs as well as in vegetative tissues. These results are consistent with results obtained for similar genes in other species.

**Figure 9.** Expression of flowering genes in different citrus organs. The vegetative shoot was obtained from a immature plant that had never produced flowers. The "–RT" panel represents a control experiment to confirm that the mRNA sample is not contaminated with DNA. *CsTUB* was used as a control gene to confirm the RT-PCR reaction worked.



As the first step towards developing a gene-based floral index, the expression of the citrus flowering genes was examined in buds from trees being used in the Ralex (a commercial GA formulation) trial at NSW Department of Primary Industries research station at Dareton in collaboration with Tahir Khurshid. The flowering behaviour of the control and Ralex-treated trees was analyzed in detail in the following spring by Tahir Khurshid as part of a Ralex trial. Photos of representative trees, from the June 18th treatment date, are shown in Figure 10 and Table 2. The Ralex-treated trees had far fewer flowers than control trees demonstrating that the Ralex treatment was effective. 2003 was an "on" year with excessive flowers numbers in control trees.

The expression of two other putative citrus flowering genes, *CsFUL* and *CsAP1*, identified in a related project on citrus fruit maturation, was also determined. Buds were collected on June 25th from control trees and Ralex-treated trees (200mL/100L) that had been treated 7 days previously. Buds were also collected on July 29th from control trees and Ralex-treated trees (200mL/100L) that had been treated 8 days previously. The results are shown in Figure 11. When compared at the level of entire buds (all three tissue types described above), the expression of all the flowering genes was similar regardless of whether Ralex was applied. In contrast, the expression of the meristem gene, *CsWUS*, increased slightly in buds from Ralex treated trees. This result suggests that the expression of flowering genes relative to the size of the meristem may be decreased following Ralex treatment. In other words, Ralex may be increasing meristem size in the dormant buds and diluting the activity of the flowering genes so that they are unable to promote flower development effectively.

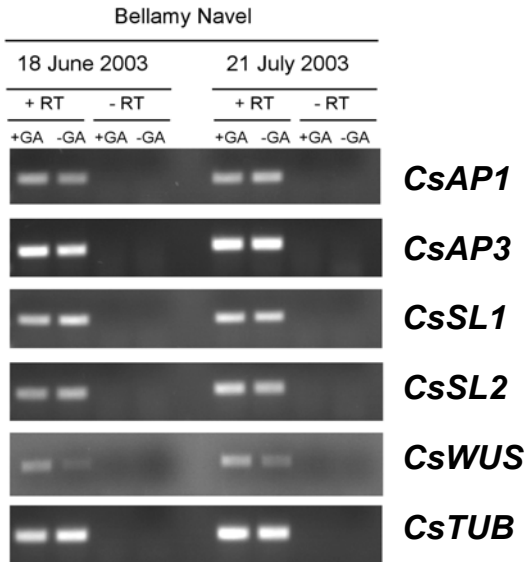
**Figure 10.** Effect of winter Ralex treatment on flowering of Bellamy Navel in the following spring. *Left*: untreated tree. *Right*: tree treated with 200 mL/100L Ralex on June 18th 2003.

**Table 2.** Ability of Ralex (200 ml/100 L) to reduce flower intensity in Bellamy Navel in the 2003 season. Data was collected in the spring following Ralex application in the preceeding winter.

| Treatment | % leafless inflorescence | % leafy inflorescence | % vegetative |
|---|---|---|---|
| Control June 18th 2003 | 34% | 11% | 55% |
| Ralex June 18th 2003 | 12% | 11% | 77% |
| Control July 21st 2003 | 52% | 13% | 35% |
| Ralex July 21st 2003 | 8% | 13% | 79% |

**Figure 11.** Expression profile of citrus MADS-box and *WUS* genes in buds harvested from trees of Bellamy Navel treated with and without Ralex (200 ml/100 L). "-RT" is a control for the RT-PCR analysis of gene expression to demonstrate that the

reaction shown in "+RT" was not contaminated with genomic DNA. *CsTUB* was used as a control gene to confirm the RT-PCR reaction worked.




In the winter of 2004, an "off" year, buds were harvested from Navelina and Washington Navel trees treated with the same dosage of Ralex as previously. In contrast to the results obtained in the "on" year of 2003, the Ralex treatments examined in this research had little effect on flowering in the "off" year of 2004 (Tables 3,4). However, as 2004 was an "off" year, the proportion of leafless inflorescences on control trees was markedly less than in 2003 (Tables 2, 3, 4).

As for the 2003 results, all of the MADS-box genes that were examined exhibited similar expression levels in buds of Ralex treated and untreated trees from both cultivars of citrus tested. Unlike the 2004 buds, little effect of Ralex on *CsWUS* expression was observed (Figure 12).

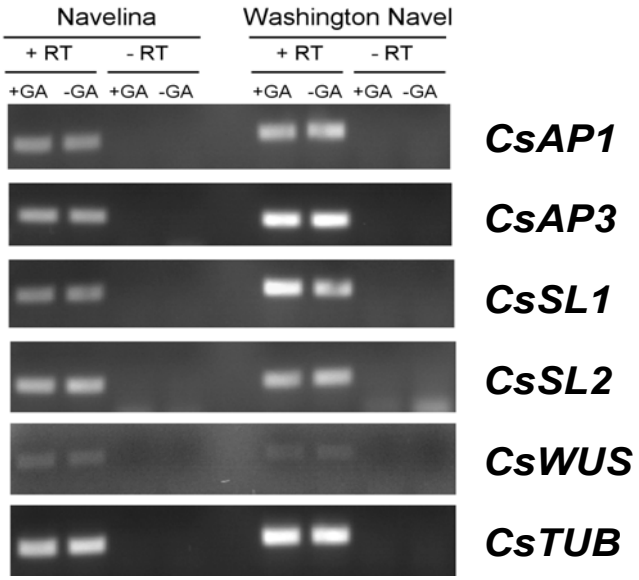
**Table 3.** Ability of Ralex to reduce flower intensity in Washington Navel in the 2004 season

| Treatment | % leafless inflorescence | % leafy inflorescence | % vegetative |
|---|---|---|---|
| Control June 22nd 2004<br>Ralex June 22nd 2004 | 22%<br>22% | 36%<br>38% | 42%<br>40% |

**Table 4.** Ability of Ralex to reduce flower intensity in Navelina in the 2004 season

| Treatment | % leafless inflorescence | % leafy inflorescence | % vegetative |
|---|---|---|---|
| Control June 22nd 2004<br>Ralex June 22nd 2004 | 6%<br>1% | 14%<br>4% | 80%<br>95% |

**Figure 12.** Expression profile of citrus MADS-box and *WUSCHEL* genes in buds harvested from trees of Navelina and Washington Navel treated with and without 200 mg/100 L Ralex (GA). The Navelina and Washington Navel buds were harvested on 29th June 2004 from trees treated with or without Ralex on 22nd June 2004. "-RT" is a control for the RT-PCR analysis of gene expression to demonstrate that the reaction shown in "+RT" was not contaminated with genomic DNA. *CsTUB* was used as a control gene to confirm the RT-PCR reaction worked.




The analyses described above were from buds harvested from trees approximately 1 week after Ralex or control treatments. However, a potential floral index would ideally predict flower numbers early in winter, for example in May before the potential application of Ralex, to allow growers to decide on appropriate Ralex applications in June or July. Consequently, immature winter buds were harvested in May, and gene expression analysed. Buds were harvested from eight different trees of Bellamy Navel located at DPI, Dareton in May 2004. These trees had previously been used for the Ralex trial in 2003 and exhibited a visible range in crop load from heavy to light. Total RNA was extracted from each sample of buds and RT-PCR was used to measure the level of expression of the selected genes (Figure 13). All the tested MADS-box genes were expressed at a similar level in buds of each of the eight trees. The expression of *CsWUS* gene, previously identified as the best candidate for analysis post Ralex treatments, was not yet detectable at this stage of bud development. Unfortunately, these results suggest that early (May) predictions of flowering using the existing genes is unlikely to be feasible, because the expression of *CsWUS* cannot be detected in buds this early in development. This is likely to be due to limitations in existing detection methodologies and the extremely small size at this early stage of the part of the bud from which flowers arise.

A key premise for the development of a citrus floral index is that individual dormant buds will exhibit marked differences in expression of flowering genes that will determine the type of shoot that subsequently arises. One explanation for the failure to observe clear differences between the expression of flowering genes in buds from Ralex and control trees is that this premise is not correct. To investigate this possibility, individual buds harvested in the 2003 season were selected from control trees and analysed. This material was selected because 2003 was an "on" year and control trees exhibited a range of floral types on individual shoots. As shown in Figure 14, only relatively subtle variation in the expression of the *CsAP1* and *CsWUS* genes was observed between individual buds.

**Figure 13.** Expression profile of citrus MADS-box and *CsWUS* genes in buds harvested from individual trees of Bellamy Navel on 20 May 2004. Trees with visibly different crop loads were selected. "-RT" is a control for the RT-PCR analysis of gene expression to demonstrate that the reaction shown in "+RT" was not contaminated with genomic DNA. *CsTUB* was used as a control gene to confirm the RT-PCR reaction worked.

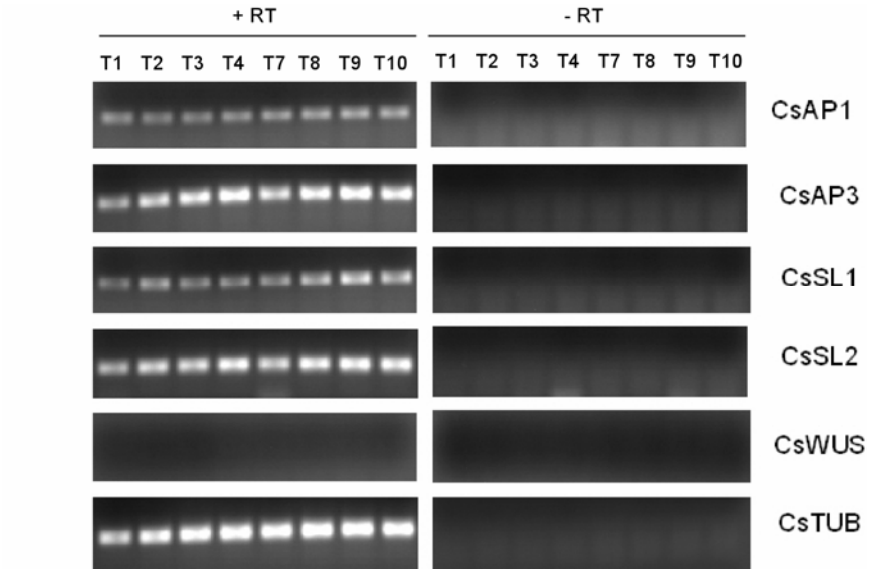
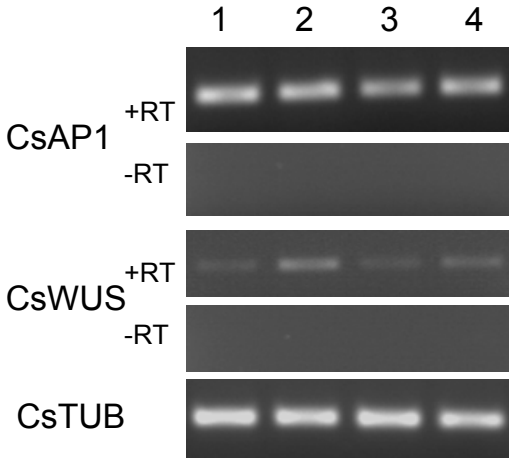


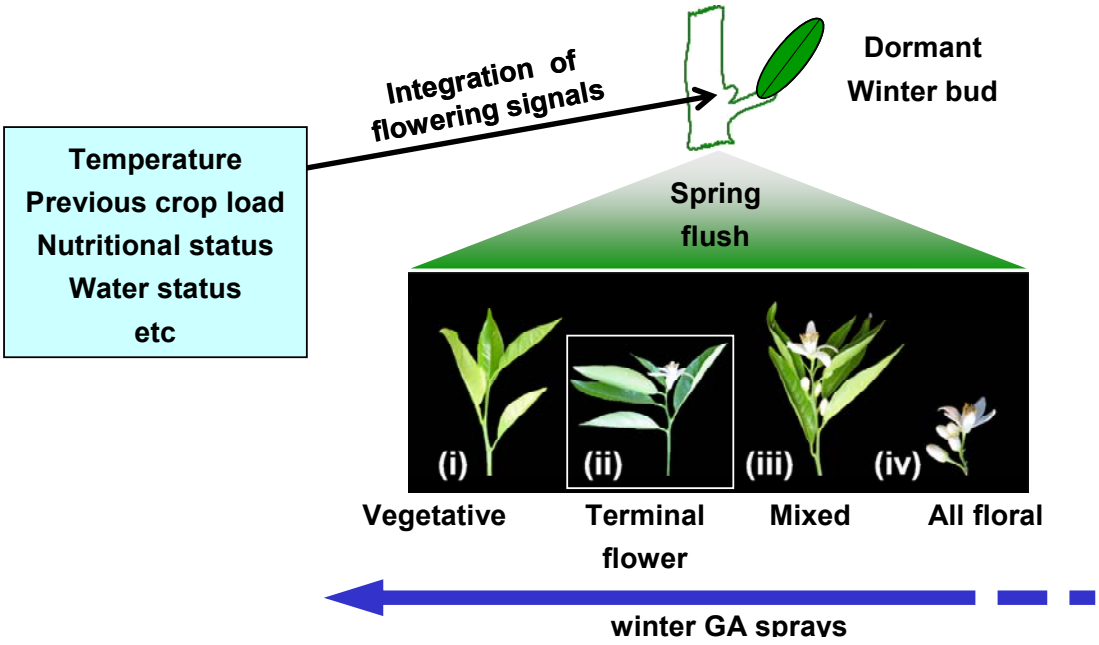

**Figure 14.** Expression profile of the citrus MADS-box gene *CsAP1* and *CsWUS* in randomly selected individual buds harvested from Bellamy Navel trees without GA treatment on 25 June 2003 (an "on" year) at Dareton. "-RT" is a control for the RT-PCR analysis of gene expression to demonstrate that the reaction shown in "+RT" was not contaminated with genomic DNA. *CsTUB* was used as a control gene to confirm the RT-PCR reaction worked.



# DISCUSSION

In mature citrus trees, four different shoot types can emerge when buds burst in spring: vegetative (no flowers), terminal flower (one flower and several leaves), mixed (several flowers and leaves) or all floral (one or more flowers with no developed leaves). The relative frequencies of the different shoot types are largely determined by the combined effects of environmental factors and the previous crop load. Winter GA sprays can be used to reduce the proportion of the "all floral" and "mixed" bud types. The single terminal flower shoot type is optimal for the production of large fruit (Figure 15).

**Figure 15.** Model of how environmental factors and the previous crop load influence shoot development in the following spring. The single terminal flower shoot type is optimal for the production of large fruit.




The overall aim of this project was to develop and test practical methods of predicting flowering intensity (a citrus floral index) in the coming spring by analysing dormant buds collected in winter for the activity of key flowering genes. This type of information would allow growers to use treatments such as winter gibberellic acid (GA) sprays more effectively to regulate flower production and hence fruit numbers and size. A prerequisite for such a predictive test is the identification of citrus flowering genes and the ability to measure their expression (i.e. activity) in dormant winter buds before possible GA treatments. This approach was chosen because simpler techniques used to predict flowering in other tree crops, such as microscopic analysis of buds or the "Merbein bunch count" for grapevines, cannot be applied to citrus prior to potential application of GA.

The function of selected citrus genes isolated in this project has been investigated by their ability to restore the floral or meristem identity defects in the corresponding Arabidopsis mutants. Based on these experiments *CsSL1* and *CsSL2* have a role in flower initiation and *CsWUS* is important for meristem development. The role of *CsAP3* has not been confirmed, and it is possible that this gene is a pseudogene that does not encode a functional protein. These physiological roles are consistent with the DNA sequence of the citrus genes and confirm that the genes controlling flowering and flower development are similar in all flowering plants. Significantly, the citrus *CsSL1* and *CsSL2* genes also affect flower organ maturation and abscission, suggesting that these genes could potentially be used to modify important traits such as fruit retention/abscission and maturation/senescence.

These results confirm that knowledge gained from genetic and molecular biology research in model plants such as *Arabidopsis* can facilitate the study of commercially important species such as citrus.

To allow comparison of the observed gene expression with flowering observed in the following spring, dormant winter buds and subsequent flowering data were obtained from the Ralex trial being conducted by Tahir Khurshid at Dareton, NSW. Buds from two years, an "on" year in 2003 and an "off" year in 2004, were analysed in detail. In buds from 2003, control trees produced a relatively high proportion of "white blossom" as indicated by the proportion of leafless inflorescences, and Ralex was very effective at reducing the number of these leafless inflorescences. By contrast, in 2004 Washington Navel and particularly Navelina produced fewer leafless inflorescences, reflecting the observation that for these trees 2004 was an "off" year. In Washington Navel the Ralex treatment analysed was ineffective at reducing flower intensity, while in Navelina Ralex had a modest effect in further reducing the already low flower intensity.

In bud samples from both years, no clear differences in the expression of flowering genes due to Ralex (200 ml/100 L) treatment were observed. This was despite the fact that Ralex was clearly effective at reducing flower numbers in 2003. A moderate change in *CsWUS* expression due to Ralex was observed in 2003, possibly suggesting that a floral index could be based on the expression of this gene. Unfortunately, *CsWUS* expression could not be detected in buds harvested in May, from trees with a range of crop loads, probably because of limitations in existing detection methodologies and the extremely small size at this early stage of the part of the bud from which flowers arise. Finally, when individual buds were examined from control trees in 2003, relatively little variation in the expression of a flowering gene (*CsAP1*) or in *CsWUS* was observed, suggesting that despite the range of shoot tissues produced in the following spring, differences in gene expression between buds are limited. This result is consistent with the expression profiles of the genes tested in pooled bud samples in which expression of several flowering genes was similar with or without Ralex treatment.

The results described above suggest that, despite success in isolating citrus flowering genes and measuring their expression in dormant winter buds, a gene-based floral index test is not feasible at the present time. There are three possible explanations for this result. Firstly, the methods available for determining gene expression may not be sufficient to distinguish small differences in gene expression. While this possibility can not be excluded, a robust floral index test that could be used in the field would need to be based on relatively large changes in gene expression to be practical. Secondly, we may not have selected to correct genes. While this possibility also cannot be formally excluded, we examined a range of genes that encode proteins likely to be acting at several stages of flowering from initial changes in meristem function (*CsSL1* and *CsSL2*) to formation of flower organs (*CsAP1* and *CsFUL*). Based on current models of flowering in several species, these genes should be representative of the processes involved in flower initiation and subsequent development.

The third possibility is that the underlying premise of the floral index test is incorrect. The key premise is that individual dormant buds will exhibit marked differences in expression of flowering genes that will determine the type of shoot that subsequently arises. An alternative hypothesis, consistent with the data presented here, is that all dormant winter buds on a mature tree have similar gene expression profiles and can potentially produce flowers. Obviously, many (vegetative) buds do not produce flowers in spring, but in this scenario flower formation is prevented despite the activity of flowering genes in winter. This hypothesis is also consistent with the observed expression of flowering genes in a vegetative shoot from a 2-year-old seedling that had never produced flowers (Figure 9), and suggests

that factors other than expression of flowering genes may limit the appearance of flowers in young trees and in many buds of mature trees. Since one of the main determinants of flowering in a mature tree is previous crop load, the most likely limiting factor, in both young and mature trees, is assimilate availability. If limited assimilate supply prevents flower formation despite the expression of flowering genes, GA application may reduce flowering by diverting assimilates to other growth processes other than flower formation. Given the established role for GAs in promoting leaf growth, and the partially antagonistic relationship between flowers and leaves illustrated in Figure 15, GA may simply act by promoting leaf growth in developing buds which in turn reduces assimilates available for flower meristems. While the above hypothesis has not yet been tested, if it is correct the implication is that the successful development of a floral index will require methods to determine overall assimilate availability to dormant buds in winter.

# TECHNOLOGY TRANSFER

**Field demonstration to growers**

Swain SM. Citrus floral index: optimising flowering and fruit size. Field presentation at Australian Citrus Growers 56th Annual Conference: Mildura, Vic.

**Oral presentations**

2 Cittgroup presentations (both at Nangiloc, Vic) in 2003/2004.
Presentation to the South Australian Citrus Board, March 2004.
2 presentations to the Murray Valley Citrus Board (providing voluntary contributions for this project) in 2003/2004.
Presentation to MVCB on 25th July 2005.

**Articles**

Swain S. (2003) Floral index: a potential diagnostic test to help reduce biennial bearing and increase citrus fruit size. CITRep ; 31, p. 9.

Article for Citrus Insight 2005: Optimising citrus fruit size by regulating flower numbers and crop load.

Steeper T. Citrus genes in action. GMO Newsletter October, 2005

**Posters**

Swain SM and Tan F-C. Citrus floral index : optimising flowering and fruit size. In: Conference Proceedings 2004: Australian Citrus Growers 56th Annual Conference: Mildura, Vic. (Australian Citrus Growers) 2004:43-44.

Tan F-C., Hooper L and Swain SM. The regulation of flowering in citrus by gibberellin (GA). ComBio, Melbourne, September 2003.

Tan F-C. and Swain SM. Isolation and characterisation of MADS-box genes from citrus. IPGSA, Canberra, September 2004.

**Refereed papers**

Tan, F-C and Swain, SM (2006) Genetics of flower initiation and development in annual and perennial plants. *submitted to Physiologia Plantarum*.

# RECOMMENDATIONS

- Future research to develop a citrus floral Index should be based on measuring assimilate availability for developing buds. Possible approaches include:
    - Determining stored carbohydrate (e.g. starch) levels directly, or
    - Measuring genes, or other factors, regulated by stored carbohydrates

- The genes identified in this project should be used to develop DNA fingerprinting for different citrus varieties. Possible uses for DNA fingerprinting include:
    - Unequivocal identification of varieties to reduce the likelihood of illegally imported material being introduced into, and grown in, Australia
    - Protection and identification of new scion and/or rootstock varieties developed in conventional breeding programs

- The genes identified in this project should be used to develop perfect molecular markers to improve seedling selection in conventional breeding

- Additional research should be undertaken on the molecular events involved in citrus flowering, as the current limited knowledge base severely limits the ability to manipulate or predict this critical physiological event.

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