(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 June 2012 (07.06.2012)

WIPO | PCT

(10) International Publication Number
WO 2012/074532 A1

(51) International Patent Classification:
A01H 5/00 (2006.01) *C12N 15/29* (2006.01)
C12N 15/82 (2006.01)

(21) International Application Number:
PCT/US2010/058916

(22) International Filing Date:
3 December 2010 (03.12.2010)

(25) Filing Language: English

(26) Publication Language: English

(71) Applicant *(for all designated States except US)*: **THE UNITED STATES OF AMERICA, AS REPRESENTED BY THE SECRETARY OF AGRICULTURE** [US/US]; 1400 Independence Ave. SW, Washington, DC 20250-0302 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **SRINIVASAN, Chinnathambi** [US/US]; 6 Putter Court, Martinsburg, WV 25405 (US). **SCORZA, Ralph** [US/US]; 29 Sallie Lane, Shepherdstown, WV 25443 (US). **CALLAHAN, Ann** [US/US]; 184 Fairmont Avenue, Shepherdstown, WV 25443 (US). **DARDICK, Chris** [US/US]; 405 Prides Crossing, Shenandoah Junction, WV 25442 (US).

(74) Agents: **GOLDBERG, Joshua, B.** et al.; The Nath Law Group, 112 S. West Street, Alexandria, VA 22314 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2012/074532 A1

(54) Title: DEVELOPMENT OF VERY EARLY FLOWERING AND NORMAL FRUITING PLUM WITH FERTILE SEEDS

(57) Abstract: To produce early flowering genotypes, plum (*Prunus domestica*) was transformed with the poplar (*Populus trichocarpa*) Flowering Locus Tl (*PtFT1*) gene. Ectopic expression of 35S::*PtFT1* Induced early flowering in vitro from transgenic plantlets within two months of transformation. When the transgenic plum plants were rooted and transferred to soil and grown in posts in the growth chamber, a number of additional lines flowered. Normal flowering and fruiting were observed in the greenhouse within one year of transformation. While dormancy was not necessary for growth or fruiting, FT plums were still winter hardy and floral bud set and flowering responded normally to changes in temperature. By manipulating a single gene, temperate tree crops can be effectively engineered for cultivation in new growing areas and for entirely new modes of agricultural production that are continuous, sustainable, and adaptable to climate change.

DEVELOPMENT OF VERY EARLY FLOWERING AND NORMAL FRUITING PLUM WITH FERTILE SEEDS

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] This invention relates to the development of transgenic plum genotypes which flower very early and continually and produce normal fruits and fertile seeds within six to twelve months and the seeds and plants obtained from such transgenic plants. The invention also relates to a method of transforming plum plant cells and plum plants utilizing a recombinant vector containing the construct comprising the gene for early flowering, *PtFT1*.

Description of the Relevant Art

[0002] *Prunus* is the horticulturally valuable genus in the family Rosaceae. Members of the family Rosaceae are cultivated for their fruits (peaches, plums, apricots, nectarines, cherries), nuts (almond) or for their ornamental flowers (flowering cherries). In addition to being a dietary supplement important for human health, *Prunus* fruits are a rich source of antioxidants which are widely reported to reduce cancer risks in humans. Conventional breeding and the application of molecular genetic technology such as structural and functional genomics and genetic engineering can be used to improve *Prunus* species. Currently, numerous genes in rosaceous fruit trees and related species have been identified through world-wide efforts of genome analyses (Retrieved from the Internet: <URL: www. bioinform.wsu.edu/gdr), but characterization of gene function through overexpression or gene silencing approaches in transgenic plants for genetic improvement of *Prunus* fruit trees is still problematic (Shulaev *et al.* 2008. *Plant Physiol.* 147: 985-1003).

[0003] Fruit tree breeding is a slow, arduous process that has changed little over the centuries. The long juvenile (pre-flowering) period of three to eight years is a severe impediment to the genetic improvement of both conventionally bred and transgenic *Prunus* fruit trees. Several generations of backcrossing and selection are required to develop improved *Prunus* cultivars; this process normally takes more than 20 years (Scorza, R. 2001. *HortScience* 36: 855-858). Limitations also include large land areas with significant field costs, and yearly limitations on flowering and fruiting related to chill and heat requirements. Shortening the 3-8 year juvenile period of *Prunus* fruit trees to a year or less by inducing

early flowering in fruit trees could dramatically reduce the time, space and cost required for genetic improvement of fruit trees and result in the production of better quality fruits.

[0004] Tree fruits are temperate crops which are cultivated in orchard systems and require a period of chilling for continued growth and fruit production. They produce a single crop of fruit/nuts per year, the timing of which depends on the species and variety but is typically between the months of June and September. The amount of chill needed for each variety and the timing of flowering and fruit set impose significant barriers to where individual species and cultivars can be productively grown. This situation results in product surplus during summer months and a lack of product in the winter months but often filled by foreign imports. The ability to alter these crops such that they are no longer limited by time of chilling and/or extend the production season through continued flowering and fruit set would provide substantial improvements to crop productivity and market delivery.

[0005] Molecular genetics of flowering has been widely reported in the model plant and ectopically overexpressed flower-inducing genes have produced early flowering in *Arabidopsis* and other herbaceous plants. Through many studies in *Arabidopsis*, the pathway to flower determination has been resolved. Normally, there is an interaction between temperature and light which affects the levels of *FLOWERING LOCUS C (FLC)* and *CONSTANS (CO)*, respectively. *FLC* negatively regulates *FLOWERING LOCUS T (FT)* and *CO* positively regulates *FT*. *FT* induces *APETALA1 (AP1), FRUITFUL (FUL)* and *SUPPRESSOR OF CONSTANS OVEREXPRESSION 1 (SOC1)*, and *SOC1* induces *LEAFY (LFY)*. Other genes associated with flower induction include: *FLOWERING LOCUS D (FD)*, and *CAULIFLOWER (CAL)*. Overexpression of these genes individually or collectively and the silencing of *TERMINAL FLOWER 1 (TFL1)*, can induce early flowering in *Arabidopsis* and other plants; for review, see Parcy, F. (2005. *Int. J. Dev. Biol.* 49: 585-593). *LFY* and *AP1* determine flower meristems (Parcy, supra); *AP1*, *LFY*, *FT* and *FUL* have been used to shorten the juvenility period in trees resulting in early flowering. Several MADS-box genes also induce early flowering in plants. Since flowering is an essential process for the survival of plant species, floral-related gene redundancy is common. *FT* is now considered as the elusive flowering signal 'florigen'. Based on the current knowledge, it appears that FT protein produced in companion cells of phloem in small veins of leaves is translocated to shoot apical meristems where it activates the shoot apical meristem-specific transcription factor *FD* which in turn recruits the meristem-identity gene *LFY* and its homologs *AP1* and *CAL* to induce flowering in plants (Abe *et al.* 2005. *Science* 309: 1052-1056; Parcy, *supra*; Wigge *et al.* 2005. *Science* 309: 1056-1059).

[0006] Several flower-inducing genes discovered in *Arabidopsis* have been tested in woody perennial plant systems. Some form of early flowering has been reported in apple, citrus, and poplar by overexpressing *MADS4* and *FT* genes. Flachowsky *et al.* (2007a. *Acta Hort.* 738: 307-312) obtained an early flowering clone of the apple cultivar 'Pinova' by over expressing a silver birch (*Betula pendula* Roth) floral meristem identity MADS-box gene, *bpMADS4*. These transgenic apple plants flowered within 13 weeks of transformation and initially produced solitary flowers, but later produced clusters of 5 flowers. Pollination of these flowers with *Malus fusca* pollen produced normal fruits and seeds (Flachowsky *et al.* 2007b. *Plant Breeding* 126: 137-145). In citrus, constitutive expression of citrus *FT (CiFT)* in trifoliated orange (*Poncirus trifoliata*) induced early flowering as early as 12 weeks after transfer of transgenic plants to a greenhouse (Endo *et al.* 2005. *Trans Res.* 14: 703-712). The transgenic lines showed variation in phenotypes such as time of first flowering and tree shape. Two *FT* genes have been isolated from poplar. *PtFT1* has been isolated from *Populus trichocarpa* (Bohlenius *et al.* 2006. *Science* 312: 1040-1043) and *PtFT2* is from *P. deltoides* (Hsu *et al.* 2006. *Plant Cell* 18: 1846-1861). Male poplar hybrid *P. tremula x P. tremuloides* flowered as early as four weeks post-transformation when overexpressing *PtFT1*, whereas *PtFT2*, which has 91% similarity in coding regions at the amino acid level, induced flowering after a year. It appears that *PtFT2* is involved in seasonal flowering. Although early flowering has been achieved by overexpressing several *MADS*-box genes and transcription factors, these genes are multifunctional and overexpression of these genes induced alterations both in vegetative and reproductive growth and development. However, *FT* is neither a transcription factor nor a MADS-box gene and overexpression of FT did not adversely affect normal growth and development of plants (Bohlenius, *supra*).

[0007] The tree fruit industry is facing challenges of climate change, reductions in available labor, the need for reduced chemical inputs, and the spread of exotic pests and pathogens. To meet these challenges the development of improved varieties is vital. The objective of our research was to utilize the knowledge of the molecular genetics of flowering gained in *Arabidopsis* and to design and implement a strategy and model system to routinely induce early flowering and normal fruiting in *Prunus*.

SUMMARY OF THE INVENTION

[0008] We have ectopically expressed the isolated *PtFT1* gene (SEQ ID NO:1) from poplar (*Populus trichocarpa*) in *Prunus domestica* and confirmed that its expression results in the induction of early and continual flowering and decreasing the long juvenile pre-flowering period in the transformed plants.

[0009] In accordance with this discovery, it is an object of the invention to provide a strategy and model system to routinely induce early and continual flowering and normal fruiting in *Prunus* using the poplar *FT* gene (*PtFT1*) in plum and to use the strategy for accelerating the *Prunus* breeding cycle to obtain new improved cultivars and for climate-independent and continual fruit production systems.

[0010] It is an object of the invention to provide transformed *Prunus* plant cells and *Prunus* plants which flower early and continually and have a shorter juvenile (pre-flowering) period wherein said plant cells and plants comprise a recombinant vector comprising the *PtFT1* gene.

[0011] It is a further object of the invention to provide seeds obtained from the *PtFT1* transgenic *Prunus* plants.

[0012] It is a still further object of the invention to provide a method of regulating time of flowering of a *Prunus* plant by ectopically overexpressing the *PtFT1* gene in a *Prunus* plant and plant cells.

[0013] It is another object of the invention to provide a method of producing an early flowering plant comprising: constructing a recombinant vector comprising the *PtFT1* gene, transforming *Prunus* plant cells with the recombinant vector, and regenerating a plant from the obtained transformant.

[0014] It is another object of the invention to provide a method of producing an early flowering plant that continually flowers and fruits and is not subject to environmental cues such as day length and/or cold-induced, heat-induced or any other environmentally or artificially-induced dormancy-promoting cues.

[0015] It is another object of the invention to provide a method of improving *Prunus* breeding to obtain new improved cultivars comprising: using a strategy for accelerating the *Prunus* breeding cycle by transforming *Prunus* plant cells with the recombinant vector comprising the *PtFT1* transgene, obtaining transformants which flower early and continually and produce ripe fruits with fertile seeds, regenerating early and continually flowering *PtFT1 Prunus* plants from the obtained transformants, breeding the *PtFT1 Prunus* transformants to

non-transformed Prunus plants to obtain improved varieties of plants, and selecting the plants that do not carry the *PtFT1* transgene for use in conventional breeding.

[0016] Other objects and advantages of this invention will become readily apparent from the ensuing description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] Figures 1A-1F depict branching and canopy architecture of the transgenic phenotype. Figure 1A shows the highly branched and profuse flowering upright transgenic phenotype. Figure 1B shows a one year old control 'BlueByrd' seedling showing few small lateral branches. Figure 1C shows the canopy of an upright grown transgenic line. Figure 1D shows the canopy of a partially upright phenotype. Figure 1E shows the bushy phenotype. Figure 1F shows the canopy architecture of the bushy phenotype. Leaves were removed from Figures 1B, 1C, and 1D to show the canopy architecture.

[0018] Figures 2A-2L depict flowering in the transgenic plants. Figure 2A depicts flower bud formation from leaf axils of an *in vitro* produced plantlet. Figure 2B depicts an *in vitro*-regenerated shoot showing a cluster of flower buds. Figure 2C depicts flowering from *in vitro*-produced transgenic plantlets. Figure 2D depicts multiple flowers produced from a plantlet. Figure 2E shows an anther from an *in vitro*-produced flower showing pollen grains. Figure 2F depicts the double pistils from an *in vitro*-produced flower. Figure 2G shows leafy sepals in flowers produced in some greenhouse-grown transgenic line. Figure 2H shows flowering in a rooted plantlet grown in a growth chamber. Figure 2I depicts early flowering from lateral shoots of a transgenic line one month after planting in the greenhouse. Figure 2J depicts development of flowers from old buds on the trunk of a transgenic plant in the greenhouse. Figure 2K shows four kinds of flowering habits of the early flowering plum phenotype. Figure 2L depicts a lateral shoot showing a single flower and terminal and axillary panicle of flowers.

[0019] Figures 3A-3F depict the development of ripe plum fruits. Figure 3A shows the development of ripe plum fruits from a pot-grown transgenic plant 6 months after planting in the greenhouse. Figure 3B shows multiple fruits from a flower. Figure 3C shows control 'BlueByrd' plums from the orchard. Figure 3D shows ripe plums developed from an early-flowered transgenic plant in the greenhouse. Figure 3E depicts a longitudinally cut control plum. Figure 3F depicts a longitudinally cut transgenic plum showing flesh and seed

development. The insert shows the size of the control seed (upper) and the transgenic plum stone.

[0020] Figure 4 depicts an early continually flowering plum plant in the greenhouse. Fig. 4A shows a mature crop on a 1-year old fruiting plant. Fig. 4B shows a close-up view of fruit of the plant of Fig. 4A. Fig. 4C depicts flowers and two fruits at different stages of development on this same plant, illustrating the continuous production of flowers and fruit on plants containing the *PtFT1* gene construct.

[0021] Figure 5 depicts quantitative PCR analyses of RNA extracted from leaves sampled from flowering and non-flowering transgenic plants grown in the greenhouse. The histogram shows relative amounts of *PtFT1* and *NPTII* transcripts in leaves as compared to transgenic line 126 anchored as 100%. Profusely flowering transgenic lines 3, 103, 158, 174, and 175 showed more transcripts as compared to non-flowering and intermittently flowering plants.

[0022] Figures 6A and 6B depict three year old, greenhouse grown plum plants. Figure 6A depicts a three year old FT plum plant with flowers and fruit that has never undergone vernalization. Figure 6B depicts a similar aged standard plum tree that has nearly ceased growing after only one year in the greenhouse without vernalization.

[0023] Figure 7 is an image showing flower panicles formed in FT plums.

[0024] Figures 8A-C show the effects of three weeks of growth at 21°C or 29°C for control plums (first two bars) and an FT plum line (2nd two bars) on flowering (Figure 8A), on bud development (Figure 8B) and on bud break (Figure 8C).

[0025] Figures 9A and 9B show the effect of temperature on fruit retention. Figure 9A shows fruit retention in plants shifted to 21°C after 6 weeks of growth at 29°C (left) vs. 21°C (right). The plant on the left carries nearly 30 fruit while the plant on the right has none. Figure 9B shows clones of the same FT plum line grown under 29°C (left) vs. 21°C (right) prior to vernalization.

[0026] Figure 10 illustrates a breeding scheme depicting the introgression of a single dominant disease resistance trait (R) from wild type germplasm with poor fruit quality to a high quality variety.

[0027] Figure 11 illustrates the short day insensitivity of FT plums.

[0028] Figure 12 illustrates the lack of chill requirement of FT plums.

DETAILED DESCRIPTION OF THE INVENTION

[0029] We have transformed Plum (*Prunus domestica* L) with the poplar (*Populus trichocarpa*) *Flowering Locus T1* (*PtFT1*) gene encoding the PtFT1 polypeptide (SEQ ID NO:1; Nilsson *et al.* U.S. Patent Application 2008/0066198 Mar. 13, 2008). This invention concerns the first occurrence of induction of early flowering in a rosaceous species, *P. domestica* by ectopic expression of *PTFT1*. This regulation of flowering in *Prunus* enables a breeding system where the limitations of juvenile period are overcome and makes possible generation times in *Prunus* of one year versus the conventional 3-10 or more years breeding cycle.

[0030] Temperate tree fruit crops require a period of dormancy to induce flower formation and bear fruit. This attribute limits their cultivation to temperate zones with sufficient chilling hours. Production is absent in the winter months and can be over-abundant during the growing season exceeding the demand of the local market and making export of fresh product difficult, particularly for fruit with poor storage qualities. Recent climate models predict that by mid-century major temperature crop production regions will no longer experience sufficient chilling to support many fruit crops and new, adaptable systems for temperate tree fruit production will be needed.

[0031] Transgenic over-expression of the *FT* gene in *Prunus domestica* resulted in trees that changed from a temperate upright tree growth habit to a bush habit capable of continual fruit bearing without the need for a period of chilling. Flowering was altered such that, instead one or a few flowers, a stem with inflorescence clusters or panicles emerged from single buds much like grapes. FT plums did not respond to cold or daylength induced dormancy but remained winter hardy in the field. Also, floral bud set and flowering responded predictably to changes in temperature. Thus, by manipulating a single gene, temperate tree crops can be effectively engineered for cultivation in new growing areas and for entirely new modes of agricultural production that are continuous, sustainable, and adaptable to climate change.

[0032] Flowering in some transgenic lines began *in vitro* within 2 months following transformation. Other lines flowered within one month after planting in the growth chamber. Plants continued to flower following transfer to the greenhouse where additional plants flowered. The intensity of flowering was positively related to the expression level of *PtFT1* mRNA. Flowers were generally fertile and produced normal fruit with viable seeds. Seedlings from these fruit also flowered *in vitro* due to constitutive high expression of *PtFT1*.

[0033] Plums usually initiate flowers in lateral buds of both the current season shoots as well as in the new growth of older spurs. Each bud contains 1-3 flowers and no leaves. All terminal buds are vegetative. However, *PtFT1*-expressing early flowering plum plants produced 1-3 axillary flower buds from leaf axils of current shoots. Terminal panicles of 4 to 8 flowers could be observed in the lateral shoots of *PtFT1*-expressing plants; this was not observed in control plum trees. The overexpression of the *PtFT1* gene altered the natural flowering habit of plums by producing terminal and axillary panicles of flowers and multiple axillary flowers. Such alteration in the natural flowering habit due to overexpression of *FT1*, *LEAFY* and *APETALA1* genes has been reported in citrus (Pena *et al.* 2001. *Nature Biotech.* 197: 263-267; Endo *et al.*, *supra*). Here, constitutive overexpression of the *PtFT1* gene increased flowering and in some cases can convert almost all the shoot apical meristem into flowers. Even the hypocotyl sections excised from the overexpressing transgenic plum zygotic embryos, when cultured on shoot regeneration medium may in some cases regenerate flower buds instead of adventitious shoots. Flowering *in vitro* can be controlled by manipulation of the *in vitro* growth medium. *In vitro* flowering of *PtFT1* expressing plantlets can be prevented by culturing in modified Quoirin and LePoivre medium containing high nitrogen and cytokinin (Quoirin and LePoivre. 1977. *Acta Hortic.* 78:437-442).

[0034] Although the *PtFT1* gene was driven by a constitutive promoter, the flowering initially occurred only in 20 percent of the plants which had accumulated comparatively high *FT* transcripts (Fig. 5). A similar large increase in *FT1* transcript prior to flower induction has been widely reported in other woody perennial plants such as grapevine (Carmona *et al.* 2002. *Plant Physiol.* 130: 68-77), apple (Wada *et al.* 2002. *Plant Mol. Biol.* 49: 566-577; Hattasch *et al.* 2008.*Tree Physiol.* 28:1459-1466), and poplar (Hsu *et al.*, *supra*). The transition from juvenile single stemmed plum seedling to a well-branched flowering adult tree takes 3 to 4 years (Scorza, *supra*), but constitutive expression of the poplar *FT1* gene in the transgenic plum plants produced several lateral branches and flowers within a month of planting in the greenhouse. This indicates that the role of the *FT1* gene is not only to induce flowering, but it appears that the *FT1* gene is involved in the phase change from juvenile to adult phase.

[0035] At a morphological level, the combination of phenotypes in FT plums rendered them distinctly different from typical Prunus species but whether or not they were capable of normal environmental transitions was not apparent. A hallmark of temperate tree crops is their dependence on seasonal cycles for growth and reproduction. Flowering is brought about by warm spring temperatures and increased day length. Then, during the long days and warm

temperatures of summer, trees enter a vegetative growth phase during which time new flower buds are set and the trees accumulate energy stores for winter. Cool weather and shortened day lengths in fall promote the onset of dormancy which is associated with terminal bud set, growth cessation, and freezing tolerance. We evaluated FT plums to determine if they could still respond to changing environmental conditions and/or undergo dormancy.

[0036] While further studies will be necessary to elucidate the effects of *PtFT1* on plum tree and flower morphology, the induction of early and continual flowering and the production of ripe fruit with fertile seeds within a year from the time of transformation presents an important genetic tool to reduce the generation interval of plum and other rosaceous fruit crops. It will facilitate rapid functional analyses of genes involved in fruit development and can be used to drastically shorten the hybridization-based breeding cycle.

[0037] "Transformation" refers to the transfer of a nucleic acid fragment into the genome of a host organism, resulting in genetically stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" organisms. Examples of methods of plant transformation include *Agrobacterium*-mediated transformation (De Blaere *et al.* 1987. *Meth. Enzymol.* 143:277) and particle-accelerated or "gene gun" transformation technology (Klein *et al.* 1987. *Nature (London)* 327:70-73; U.S. Pat. No. 4,945,050, incorporated herein by reference). Additional transformation methods are disclosed below. Thus, isolated polynucleotides of the present invention can be incorporated into recombinant constructs, typically DNA constructs, capable of introduction into and replication in a host cell. Such a construct can be a vector that includes a replication system and sequences that are capable of transcription and translation of a polypeptide-encoding sequence in a given host cell. A number of vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants have been described in, *e.g.*, Pouwels *et al.* 1985. Supp. 1987. *Cloning Vectors: A Laboratory Manual*; Weissbach and Weissbach. 1989. *Methods for Plant Molecular Biology,* Academic Press, New York; and Flevin *et al.* 1990. *Plant Molecular Biology Manual*, Kluwer Academic Publishers, Boston. Typically, plant expression vectors include, for example, one or more cloned plant genes under the transcriptional control of 5' and 3' regulatory sequences and a dominant selectable marker. Such plant expression vectors also can contain a promoter regulatory region (*e.g.*, a regulatory region controlling inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

[0038] As used herein, the terms “nucleic acid molecule”, "nucleic acid sequence", "polynucleotide", "polynucleotide sequence”, "nucleic acid fragment", "isolated nucleic acid fragment" are used interchangeably herein. These terms encompass nucleotide sequences and the like.

[0039] The term "isolated" polynucleotide refers to a polynucleotide that is substantially free from other nucleic acid sequences, such as other chromosomal and extrachromosomal DNA and RNA, that normally accompany or interact with it as found in its naturally occurring environment. However, isolated polynucleotides may contain polynucleotide sequences which may have originally existed as extrachromosomal DNA but exist as a nucleotide insertion within the isolated polynucleotide. Isolated polynucleotides may be purified from a host cell in which they naturally occur. Conventional nucleic acid purification methods known to skilled artisans may be used to obtain isolated polynucleotides. The term also embraces recombinant polynucleotides and chemically synthesized polynucleotides.

[0040] As used herein, "recombinant" refers to a nucleic acid molecule which has been obtained by manipulation of genetic material using restriction enzymes, ligases, and similar genetic engineering techniques as described by, for example, Sambrook *et al.* 1989. Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY or DNA Cloning: A Practical Approach, Vol. I and II (Ed. D. N. Glover), IRL Press, Oxford, 1985.

[0041] A “construct” or “chimeric gene construct” refers to a nucleic acid sequence encoding a protein, here the PtFT1 protein, operably linked to a promoter and/or other regulatory sequences.

[0042] As used herein, the term "express" or "expression" is defined to mean transcription alone. The regulatory elements are operably linked to the coding sequence of the *PtFT1* gene such that the regulatory element is capable of controlling expression of *PtFT1* gene. "Altered levels" or "altered expression" refers to the production of gene product(s) in transgenic organisms in amounts or proportions that differ from that of normal or non-transformed organisms.

[0043] As used herein, the terms "encoding", “coding”, or "encoded" when used in the context of a specified nucleic acid mean that the nucleic acid comprises the requisite information to guide translation of the nucleotide sequence into a specified protein. The information by which a protein is encoded is specified by the use of codons. A nucleic acid encoding a protein may comprise non-translated sequences (*e.g.*, introns) within translated

regions of the nucleic acid or may lack such intervening non-translated sequences (*e.g.*, as in cDNA).

[0044] The term "operably linked" refers to the association of two or more nucleic acid fragments on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (*i.e.*, that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

[0045] "Regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

[0046] "Promoter" refers to a nucleotide sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. The promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. Accordingly, an "enhancer" is a nucleotide sequence that can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic nucleotide segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. The tissue-specificity of a promoter, for example, is exemplified by the promoter sequence (described above) which specifically induces gene expression in root tips. Promoters that cause a nucleic acid fragment to be expressed in most cell types at most times are commonly referred to as "constitutive promoters". New promoters of various types useful in plant cells are constantly being discovered; numerous examples may be found in the compilation by Okamuro and Goldberg. 1989. *Biochemistry of Plants* 15:1-82. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, nucleic acid fragments of different lengths may have identical promoter activity.

[0047] "RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complementary copy

of the DNA sequence, it is referred to as the primary transcript or it may be an RNA sequence derived from posttranscriptional processing of the primary transcript and is referred to as the mature RNA. "Messenger RNA (mRNA)" refers to the RNA that is without introns and that can be translated into polypeptides by the cell. "cDNA" refers to a DNA that is complementary to and derived from an mRNA template. The cDNA can be single-stranded or converted to double stranded form using, for example, the Klenow fragment of DNA polymerase I. "Sense" RNA refers to an RNA transcript that includes the mRNA and so can be translated into a polypeptide by the cell. "Antisense", when used in the context of a particular nucleotide sequence, refers to the complementary strand of the reference transcription product. "Antisense RNA" refers to an RNA transcript that is complementary to all or part of a target primary transcript or mRNA and that blocks the expression of a target gene. The complementarity of an antisense RNA may be with any part of the specific nucleotide sequence, i.e., at the 5' non-coding sequence, 3' non-coding sequence, introns, or the coding sequence. "Functional RNA" refers to sense RNA, antisense RNA, ribozyme RNA, or other RNA that may not be translated but yet has an effect on cellular processes.

[0048] A "protein" or "polypeptide" is a chain of amino acids arranged in a specific order determined by the coding sequence in a polynucleotide encoding the polypeptide. Each protein or polypeptide has a unique function.

[0049] It is to be understood that as used herein the term "transgenic" includes any cell, cell line, callus, tissue, plant part, or plant the genotype of which has been altered by the presence of a heterologous nucleic acid including those transgenics initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic. The term "transgenic" as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by conventional plant breeding methods or by naturally occurring events such as random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition, or spontaneous mutation.

[0050] As used herein, the term "plant" includes reference to whole plants, plant organs (*e.g.*, leaves, stems, roots, *etc.*), seeds, plant cells, and progeny of same. Parts of transgenic plants are to be understood within the scope of the invention to comprise, for example, plant cells, protoplasts, tissues, callus, embryos as well as flowers, stems, fruits, leaves, roots originating in transgenic plants or their progeny previously transformed with a DNA molecule of the invention and therefore consisting at least in part of transgenic cells, are also an object of the present invention.

[0051] As used herein, the term "plant cell" includes, without limitation, seeds suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores. The class of plants that can be used in the methods of the invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants.

[0052] The successful transformation of *Prunus* with *PtFT1* is a major step in overcoming generation time in fruit trees and will aid in devising new strategies for improving breeding in *Prunus*, thus ensuring the development of improved varieties of *Prunus*.

[0053] The creation of plum trees that do not undergo dormancy and produce flowers and fruit continually offer new strategies for growing and producing prunus fruits/nuts in a fashion that is continual and climate-independent and will provide a more stable and continuous supply of these products and their derivatives.

EXAMPLES

[0054] Having now generally described this invention, the same will be better understood by reference to certain specific examples, which are included herein only to further illustrate the invention and are not intended to limit the scope of the invention as defined by the claims.

EXAMPLE 1

Transformation

[0055] The plasmid pK2GW7 containing the Cauliflower Mosaic Virus 35S (CaMV 35S) promoter, and nptII and PtFT1 genes (Nilsson *et al.*, *supra*) were transformed into the *Agrobacterium tumefaciens* strain GV3101. Hypocotyl sections excised from surface-sterilized mature seed embryos of plum cultivar 'BlueByrd' were transformed with *A. tumefaciens* strain GV3101 containing the *35S::PtFT1* gene (Bohlenius *et al.*, *supra*). The transformed hypocotyl sections were cultured *in vitro* and transgenic plants were regenerated and rooted as described in Petri *et al.* (2008. *Mol. Breeding* 22:581-591). The rooted plantlets were acclimated in the growth chamber (20°C; light intensity 70 µmol photons m^2s^{-1}, 16 h light/8 hr dark photoperiod) for 2-3 weeks and planted in 6 or 9 inch pots and grown in a temperature controlled (27°C) glasshouse under sunlight during the months of December 2007 to November 2008 to evaluate flowering and fruit production.

EXAMPLE 2

Transgenic Plants: Vegetative and Flowering Characteristics

[0056] The presence of *35S::PtFT1* gene in plum was determined by PCR analyses of DNA extracted from leaves using the published primers (Bohlenius *et al.*, *supra*). A total of 196 transgenic plum plants representing 56 transgenic clones were regenerated. Flowering was scored *in vitro*, in the growth chamber, and in the greenhouse. Since 'BlueByrd' plum is self incompatible, pollen from compatible plum cultivars 'Stanley' or Cacanska Lepotica' was used to pollinate the transgenic plum flowers in the greenhouse. Fully ripe plum fruits were evaluated for size, color, brix, and stone and seed development. Viability of transgenic seed embryos was determined by culturing embryo shoot tips and hypocotyls sections following the tissue culture methods of Petri *et al.* (*supra*) without the use of antibiotic selection.

[0057] Extensive lateral shoot production was observed in all transgenic plants within 6 months of planting in the greenhouse (Fig. 1, Table 1). Non-transgenic control plants produced few small lateral shoots even after a year of growth (Fig. 1B). While most transgenic plants were branched and grew upright (Figs. 1A and 1C), some transgenic plants showed a combination of upright and bushy growth (Fig. 1D). Still others of the transgenic plants were bushy and recumbent resembling a ground cover plant due to early lateral branching and the lack of apical dominance of the main shoot (Figs. 1E and 1F). Unlike flowering, profuse lateral branching occurred even in plants which expressed low levels of *PtFT1* transcript.

Table 1. Production of lateral shoots and flowers in *PtFT1*-expressing transgenic plum plants after 10 months of growth and development in nine inch pots under the greenhouse conditions.

A. Upright and Semi-upright Phenotypes						
Transgenic Line	Flowering Time In Months	Number of Lateral Shoots	Number of Flowers Per Leaf Axil			
			1	2	3-5	Terminal Panicle
3	4	28	109	32	18	17
16	9	18	2	0	0	0
27	10	12	4	3	2	0
29	8	16	8	1	2	0
32	8	18	4	2	0	0
33	10	15	1	0	0	0
34	7	27	83	23	4	2
51	10	11	1	0	0	0
52	10	11	1	0	0	0
73	8	17	2	1	0	0
74	9	15	0	0	3	0
76	8	10	1	4	1	0

88	8	16	4	3	1	0
90	7	17	6	3	0	0
91	7	21	4	3	0	0
103	1	33	79	52	53	21
117	6	12	4	2	1	0
118	7	11	2	5	0	0
119	10	14	2	0	0	0
139	10	11	5	4	2	0
140	7	18	5	3	1	0
147	7	13	2	0	0	0
148	7	11	2	2	0	0
152	7	12	4	2	0	0
157	5	26	24	11	12	4
175	7	19	3	2	1	0
183	5	13	8	3	0	0
187	6	18	1	2	1	0
188	6	16	3	1	0	0
189	6	6	3	0	0	0
192	7	12	5	0	0	0
193	7	13	3	0	0	0

B. Bushy Phenotypes

Transgenic Line	Flowering Time In Months	Number of Lateral Shoots	Number of Flowers Per Leaf Axil			
			1	2	3-5	Terminal Panicle
52	7	15	2	1	0	0
107	7	23	4	1	0	0
126	6	32	5	7	0	0
141	6	12	3	0	0	0
158	6	28	18	12	3	3
174	5	21	31	22	8	3
222	7	9	1	1	0	0
223	8	14	7	1	0	0

[0058] The FT plum shrub habit appeared to be the result of three characteristics: 1) Loss of apical dominance that was sometimes, but not always, due to the production of a terminal inflorescence, 2) lateral branches tended to have weaker attachment sites and repeatedly cracked and re-healed eventually resulting in downward branch angles, and 3) the trunk and lateral branches did not grow straight and instead curved. Second, FT plum lines grew continuously and did not consistently set terminal buds unlike non-transformed control trees that undergo growth cessation as day lengths become shorter. Some FT plum lines continued to grow, flower, and produce fruit even after 3 years at 65-85°C in a temperature controlled greenhouse.

[0059] Although lateral branching occurred even at a low level of *PtFT1* gene expression, induction of flowering requires accumulation of more than a certain threshold level of *FT1* transcript. Flower buds were produced *in vitro* from about four percent of transgenic plum plantlets within two months of transformation. Few *in vitro*-produced flower buds produced fully developed flowers with normal calyx, corolla and pollen-bearing anthers, but they had

2-4 pistils (Figs. 2A-2C). Up to three flower buds developed from each leaf axil (Fig. 2A). Clusters of flower buds developed from some shoots (Fig. 2B), but these flower bud clusters atrophied without developing further. Most of the *in vitro* flowering plantlets lacked vegetative shoot meristems and therefore could not be rooted and planted in the soil for further evaluation. Plants that did not flower *in vitro* were rooted and transferred to the growth chamber (400 μ Einstein light intensity; 16/8 photoperiod; 21-24 °C temperature; 75% humidity) where six plants flowered within a month of being transferred to the growth chamber. These produced normal flowers, but these flowers did not set fruits probably due to lack of cross pollination (Fig. 2H). Up to five flowers were produced per plantlet (Fig. 2D).

[0060] Following transfer to the greenhouse 20.9% of plants (41 plants out of 196 plants planted) flowered between 1 month (Fig. 2I) and 10 months after planting in the greenhouse (Figs. 1A and 1E, Table 1). The temperature in the glass house varied from 24 to 27 °C and the light was natural sun light. These included those plants that had flowered in the growth chamber. The frequency of flower formation varied. Of the 41 flowering plants from 56 lines, five plants (transgenic lines 3, 103, 34, 158, and 174) produced flowers profusely for 4-6 weeks, while others produced few flowers sporadically for 6 weeks (Table 1). Accumulation of *PtFT1* transcript in leaves was high in profusely flowering plants as compared to sparsely flowering plants. Non-flowering *PtFT1* plants accumulated the lowest levels of transcript in leaves.

[0061] Flowers generally formed in the leaf axils of lateral shoots (Figs. 2I and 2L). The number of flowers per leaf axil varied from 1 to 3 (Fig. 2K, Table 1). Some leaf axils bore both vegetative and flower buds. In addition to axillary flowers, lateral shoots often produced a terminal panicle of 4 to 8 flowers (Figs. 2K and 2L). Most flowers had 5 sepals, 5 petals, 16 to 23 anthers and 2 to 4 pistils (Figs 2E and 2F). Flowers also appeared from old buds on the trunk. These flowers also produced fruits following hand pollination (Fig. 2J). The majority of flowers showed normal morphology with the exception of a higher number of pistils per flower (Figs. 2H, 2K,, and 2L); however, a few flowers were abnormal, *i.e.*, they had leafy overgrown sepals (Fig. 2G) or malformed petals, 4, 6, or 7 petals or few anthers.

[0062] A total of 32 plum fruits were produced in the greenhouse by hand pollinating the flowers in the profusely flowering transgenic lines. Pollination of multiple pistils produced up to 3 ripe fruits per flower (Fig. 3 B). Plum fruits in the greenhouse-grown transgenic plants developed normally and ripened 5 months after fruit set. Ripe fruits displayed red purple skin color (Fig. 3D; Table 2) and greenish-yellow flesh color (Fig. 3F). Fruits were smaller than the fruits produced from plum trees grown in the orchard (Figs. 3 C and 3D).

The size of fruits varied from 10 to 37 mm in length and 5 to 34 mm in diameter (Table 2a, b). Early continually flowering 1-year old fruiting plants produce mature fruit crops in the greenhouse. Flowers and fruits at different stages of development are found on the same plant (Fig. 4C). Brix of fruit juice varied from 8 to 11°.

Table 2a. Characteristics of fruits, stones, and seeds harvested from an orchard-grown control plum tree and from the pot-grown *PtFT1*-expressing transgenic plum plants in the greenhouse.

Transgenic Line	Fruit (mm)		Stone (mm)		Seed (mm)		Brix Degree	Fruit Color*	
	L	D	L	W	L	W		External	Internal
Control	44.8	37.8	27.2	14.8	16.2	10.4	15.6	Violet	Green-Yellow
3	31.7	29.1	14.4	7.2	10.0	5.0	10.5	Blue 106B	Green-Yellow
103	36.8	33.8	16.8	9.3	14.4	8.2	9.9	Blue 106D	Green-Yellow

* Fruit Color was determined by using Royal Horticultural Society Color Chart, UK
L – Length; D – Diameter; W – Width

Table 2b. Characteristics of fruits, stones, and seeds harvested from an orchard-grown control plum tree and from the pot-grown *PtFT1*-expressing transgenic plum plants in the greenhouse.

Transgenic Line	Number of Fruits	Fruit Diameter mm	Fruit Weight gm	Brix
Control	5	37.8	45.0	15.6
#3	18	28.5	N.D.	13.1
#29	6	30.1	18.1	11.5
#32	3	30.0	15.3	Mealy, no juice
#34	3	33.3	21.6	12.3
#88	1	37.0	33.9	mealy
#90	2	31.0	16.9	11.8
#91	9	34.7	25.1	12.0
#103	9	31.8	ND	10.2
#152	1	28.0	13.4	10.8
#157	21	31.0	17.0	10.4

N.D. Not Determined

[0063] Stones and seeds developed normally, but they were smaller than the orchard-grown plum seeds (Fig. 3F insert). Embryos excised from the seeds of early fruiting transgenic plums readily germinated *in vitro*. The hypocotyl sections excised from transgenic seed embryos regenerated adventitious shoots *in vitro* when the regeneration protocols of Petri *et al.* (*supra*) were utilized, without antibiotic selection. Some of these adventitious shoots and seedlings also produced flower buds *in vitro*, but these flower buds seldom develop into flowers. However, high levels of *PtFT1* expression appeared to affect formation of vegetative meristem in the succeeding generation of transgenic plants developed from seed of profusely flowering lines. When
the shoot apices of profusely flowering and fruiting transgenic lines, *i.e.*, PtFT lines 3, 34, 103, and 157, were cultured, most of the *in vitro*-multiplied shoots produced up to 10 flowers per plantlet and these plants had few vegetative meristems. Rooting of these plantlets was also slow compared to control plantlets.

[0064] Alternatively, seeds were extracted from the stony endocarp by cracking open the endocarp. Seeds were then soaked in a 1.2% solution of Sodium hypochlorite for 20 min to 2 hrs then soaked in a solution of 500 ppm benzyladenine and 500 ppm gibberelllic acid for 8-16 hrs. The seed coat may then be stripped off, but it is not necessary to strip off the seed coat. Seeds germinate within days of this treatment, thus avoiding a lengthy stratification requirement (200 – 1500 hrs at 4 °C) that *Prunus* seed normally need for germination. This procedure also avoids the need for *in vitro* culture of embryos.

EXAMPLE 3

Transgenic Plants: Effects of Induced Dormancy

[0065] Three FT plum lines were clonally propagated and placed in environmental growth chambers with non-transgenic controls at either 21°C or 29°C for 8 weeks. Growth rate, node number, bud development or formation, bud break, and flower number were measured at 2 week intervals (Fig. 8). Data showed that growth rate, node number, and rates of bud break were similar in both temperatures but both node number and bud break were higher for FT lines relative to controls. In contrast, flowering was more prolific in FT plums at 21°C while higher numbers of floral buds were set at 29°C. To confirm the temperature effect, after 8 weeks five plants were swapped from each chamber. Again flowering increased in plants shifted to 21°C and was repressed at 29°C. Abnormal flowers only developed at 29°C even in buds set at 21°C suggesting that de-differentiation occurred post bud break.

[0066] FT plums grown in the greenhouse were insensitive to short days during the winter months and did not undergo growth cessation. A controlled experiment was performed for FT plums in the growth chamber under short (8 hr) and long (16hr) day lengths. After six weeks growth rate, bud set, bud break, and flower number were measured and showed no significant difference between the two light regimes unlike control plums which showed decreased growth rate under short days (Figure 11).

[0067] Dormancy is a complex state and occurs as a consequence of diverse signaling pathways. A key characteristic of dormant temperate trees is the requirement for a sufficient number of chilling hours (defined as hours exposed to 0 - 7 °C) before efficient vernalization can occur. Chilling time varies among species and cultivars but for *P. domestica* a minimum of 800-1,000 chilling hours is typically required. We tested clonally propagated individuals from two FT plum lines along with an equal number of controls. Plants were placed in cold storage at 5°C, moved to the greenhouse at one week intervals, and the time to bud break was measured. Results showed that cold temperature treatment lead to a marked delay in bud break in control trees but not in FT plums (Figure 12).

[0068] While laboratory experiments indicated that FT plums could respond normally to environmental cues and enter dormancy, we wanted to test whether they could survive a natural winter environment. Four plants each from two different FT plum lines along with controls were planted in field plots in late summer 2009. In late January, bud sticks were removed and checked for bud survival rates. Line #34 had 100% survival while line #3 was 84%. In spring 48 out 50 FT plum lines survived and resumed growth.

EXAMPLE 4

RNA Extraction

[0069] One leaf punch from a single paper punch was collected from three different fully expanded, non-waxy leaves from one plant and pooled into one sample (~ 10 mg total), frozen in liquid nitrogen and stored at – 80°C until processed. All the leaf samples for one experiment were collected between 13:00 and 14:30 of the same day. RNA was extracted from the leaf material using the MagMAX-96 Total RNA Isolation Kit (Applied Biosystems, Foster City, CA) with some modifications to the protocol. Basically 100 µl of Lysis/Binding Solution was added to the sample along with ¼ amount of Lysing Matrix D (BIO 101 Systems, Thermo Scientific, Waltham, MA) and processed in a FastPrep (FP120, BIO 101, Thermo Scientific) bead beater for 27 sec at a 5 speed setting. The beaded material was spun

first, and then the supernatant was placed in a clean microcentrifuge and processed as described in the manufacturer's protocol except that two washes were performed at each step. Three µl of the RNA was evaluated on a gel.

EXAMPLE 4

Quantitative Real-time PCR

[0070] Quantitative Real-time PCR (qRT PCR) was performed on the RNAs utilizing a one-step protocol with RNAse Inhibitior (Applied Biosystems), MuLv Reverse Transcriptase (Applied Biosystems) and SYBRGreen PCR Master Mix (Applied Biosystems) following the manufacturer's protocol. Initially, all the RNAs were run as a single reaction with either primers for chlorophyll A/B binding protein or the PFT transgene, with and without the RT, in order to verify the lack of significant DNA contamination. To determine the relative levels of RNA expressed, the RNAs were diluted (0.33 µl/reaction), and run in triplicate in 10 µl reactions on an ABI7900 (ABI). Primer sequences are listed in Table 3. All RNAs were also run with 26S primers at an additional 1000 fold dilution to determine the relative amount of each RNA in the reactions. A standard curve was run in triplicate with each primer set to determine the relative amounts. The results of the triplicate reactions were averaged and normalized by the relative amount of 26S RNA. To keep all the numbers on the same scale, the level of expression of line 126, the low flowering line, was set at 100% and all the other lines were compared to that line.

Table 3. Primer Sequences used:

PRIMER	SEQUENCE	SEQ ID NO:
1PtFT1-5'	CAGAACTTCAACACCAGAGA	3
1PtFT1-3'	TCCTACCACCAGAGCCACT	4
2PtFTb-5'	TTCTACACTCTGGTTATGGTGGACC	5
2PtFTb-3'	GTTGCCGAAACAAGACGAAAAC	6
326S-5'	GCAGCCAAGCCTTCATAGCG	7
326S-3	GTGCGAATCAACGGTTCCTC	8
42057-5'	GTGTTCAGACCACTTCCTTCATCC	9
42057-3'	CCATCTTCAACCTTCGGCTTC	10
54040-5'	CAAGGCAACTACAACTCAGGCAG	11
54040-3'	AGGCATCCCATACATAACACCAAG	12

1 PtFT1 (415-517) Bohlenius *et al.* 2006. *Science* 312:1040.
2 Designed from Acc# DQ387859 (187-379)
3 Previously published (Moon and Callahan. 2004.)
4 Wu *et al.* 2006. *Genetics* 174:1407-1420.
Designed from Contig 2057 in the Prunus Assembly V4
http://www.bioinfo.wsu.edu//cgi-bin/gdr/gdr_EST_contig_search.cgi?genus=Prunus.
5 As above, but from Contig 4040.

[0071] The ability of FT plums to continuously produce fruit irrespective of day length or chilling time and still survive extreme cold suggests they could be grown in both tropical and temperate climates. Their shrub growth habit and continual fruiting makes them suitable for non-orchard production systems either in the field or protected facilities where artificial changes in light and temperature could be used to manipulate growth and maximize fruit production to meet off season demand. Thus, FT plums represent an important milestone in temperate tree fruit biotechnology and pave the way for future advances to address the challenges facing temperate tree crop agriculture.

[0072] All publications and patents mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

[0073] The foregoing description and certain representative embodiments and details of the invention have been presented for purposes of illustration and description of the invention. It is not intended to be exhaustive or to limit the invention to the precise forms disclosed. It will be apparent to practitioners skilled in this art that modifications and variations may be made therein without departing from the scope of the invention.

CLAIMS

We claim:

1. A transgenic *Prunus* plant in which the *PtFT1* cDNA has been introduced or a progeny of said plant in which said cDNA has been introduced and in which overexpression of *PtFT1* in said plant or progeny of said plant exhibits early flowering and a shortened juvenile period.

2. The transgenic *Prunus* plant of Claim 1 in which overexpression of *PtFT1* in said plant or progeny of said plant also exhibits continual flowering and production of fruit.

3. The transgenic *Prunus* plant of Claim 2 in which overexpression of *PtFT1* in said plant or progeny of said plant also exhibits a compact, shrub-type architecture.

4. A transgenic *Prunus* plant in which the *PtFT1* cDNA has been introduced or a progeny of said plant in which said cDNA has been introduced and in which overexpression of *PtFT1* in said plant or progeny of said plant enables fruit production in a broader range of climates.

5. A transgenic *Prunus* plant in which the *PtFT1* cDNA has been introduced or a progeny of said plant in which said cDNA has been introduced and in which overexpression of *PtFT1* in said plant or progeny of said plant exhibits new flowering structures.

6. A transgenic *Prunus* plant in which the *PtFT1* cDNA has been introduced or a progeny of said plant in which said cDNA has been introduced and in which overexpression of *PtFT1* in said plant and progeny of said plant result in PtFT1 lines which segregate both early flowering and non-flowering progeny wherein said non-flowering progeny do not carry any transgenes.

7. A plant cell, a plant part, or a plant tissue of the plant of any one of Claims 1-6.

8. The transgenic plant according to any one of Claims 1-6, wherein said *PtFT1* cDNA encodes a polypeptide identified by SEQ ID NO:2.

9. A plant part from the transgenic plant according to any one of Claims 1-6, wherein the plant part contains *PtFT1* cDNA.

10. A transgenic seed of the transgenic *Prunus* plant according to Claim 1.

11. The seed of Claim 10, wherein the seed carrying the *PtFT1* gene insert is true breeding for early flowering and shortened juvenile period as compared to a wild type variety of the seed.

12. A recombinant construct comprising the *PtFT1* cDNA and one or more regulatory elements operatively linked to said nucleic acid wherein ectopic overexpression of said cDNA in plant cells results in early flowering in said plant cells.

13. The recombinant construct of Claim 12 wherein said regulatory element is a constitutive, inducible, or tissue-specific promoter operably linked to said nucleotide sequence.

14. The recombinant construct of Claim 13 wherein said regulatory element is the CaMV 35S promoter.

15. A vector comprising the construct of Claim 12.

16. A host cell comprising the vector of Claim 15.

17. The host cell of Claim 16, wherein said host cell is a plant cell.

18. A method of regulating early flowering in a *Prunus* plant comprising introducing the *PtFT1* cDNA according to Claim 12 into a *Prunus* plant or *Prunus* plant cells and then allowing said nucleic acid to be expressed in said plant or plant cells.

19. A method of making an early and continually flowering *Prunus* plant, said method comprising: providing a *Prunus* plant cell capable of regeneration; transforming said plant cell with a construct comprising *PtFT1* cDNA according to Claim 12 or with a vector comprising said construct according to Claim 15; culturing said plant cell under plant cell growing conditions; regenerating from said plant cell a plant; and observing expression of said cDNA molecule in said plant, whereby the cDNA molecule is expressed at a level sufficient to make an early flowering plant.

20. A method of providing continual, year round *Prunus* breeding to obtain new improved cultivars comprising: accelerating the *Prunus* breeding cycle by transforming *Prunus* plant cells with the recombinant vector comprising the *PtFT1* transgene, obtaining transformants which flower early and continually and produce ripe fruits with fertile seeds, regenerating early and continually flowering *PtFT1 Prunus* plants from the obtained transformants, breeding the *PtFT1 Prunus* transformants to non-transformed *Prunus* plants to obtain improved varieties of plants.

21. A method of improving *Prunus* breeding to obtain new improved cultivars comprising: accelerating the *Prunus* breeding cycle by transforming *Prunus* plant cells with the recombinant vector comprising the *PtFT1* transgene, obtaining transformants which flower early and continually and produce ripe fruits with fertile seeds, regenerating early and continually flowering *PtFT1 Prunus* plants from the obtained transformants, breeding the *PtFT1 Prunus* transformants to non-transformed *Prunus* plants to obtain improved varieties of plants, and selecting the plants that do not carry the *PtFT1* transgene for use in conventional breeding.

22. A method of providing continuous *Prunus* fruit production in protected structures such as a greenhouse or high tunnels comprising: accelerating the *Prunus* breeding cycle by transforming *Prunus* plant cells with the recombinant vector comprising the *PtFT1* transgene, obtaining compact, shrub-type transformants which continuously flower and produce fruit and which can be grown in large numbers in an enclosed space.

23. A method of providing continuous *Prunus* fruit production in a broad range of climates.

24. A method of generating *Prunus* plants wherein said plants exhibit new flowering structures.

25. A strategy for FT plum propagation that ensures control of flowering, bud set, bud break, and fruit set in FT plums.

26. A method of rapidly screening fruit/flower phenotypes in function genomics screens.

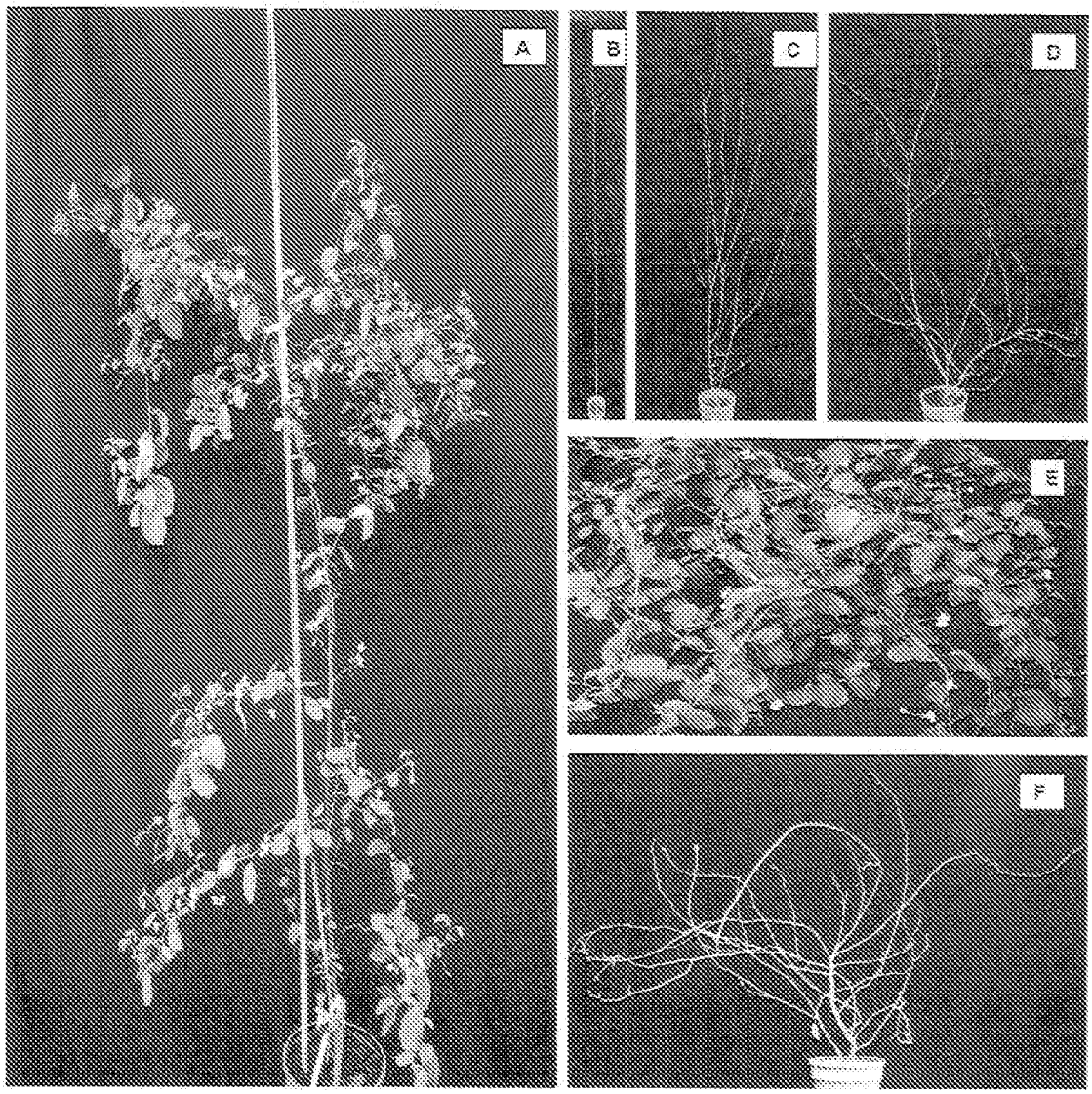


Fig. 1A-F

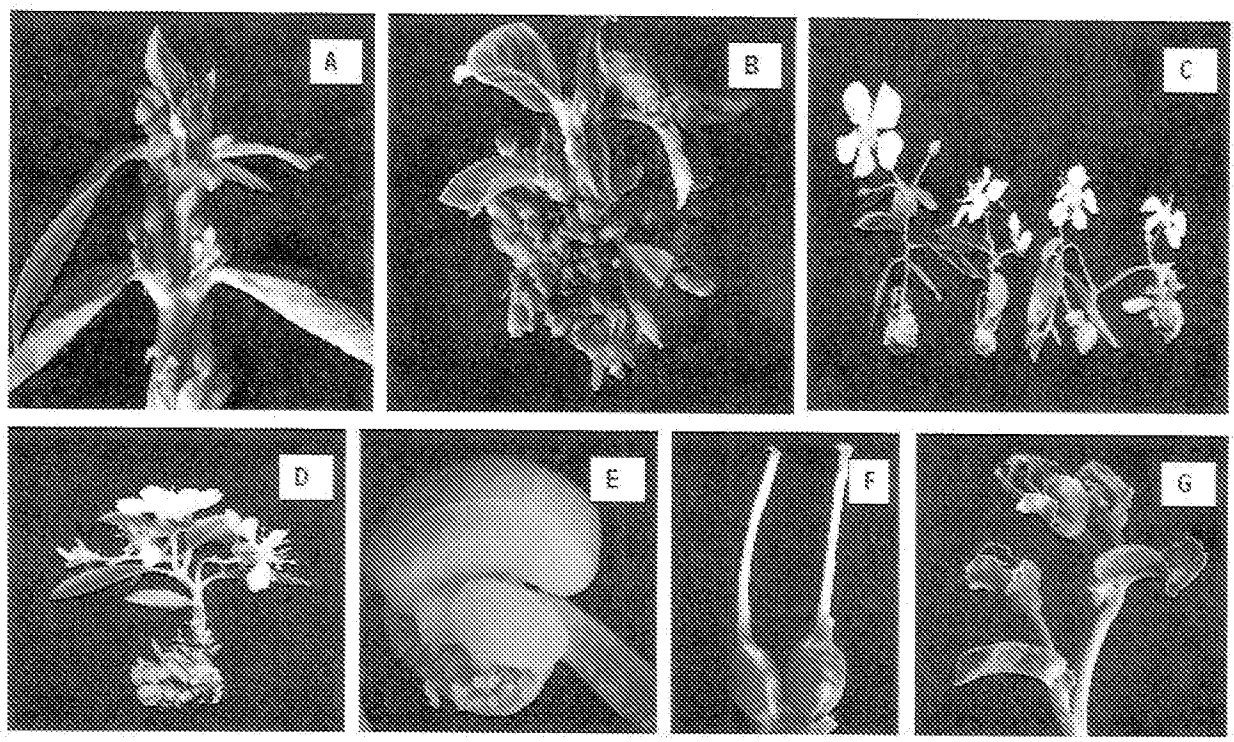


Fig. 2A-G

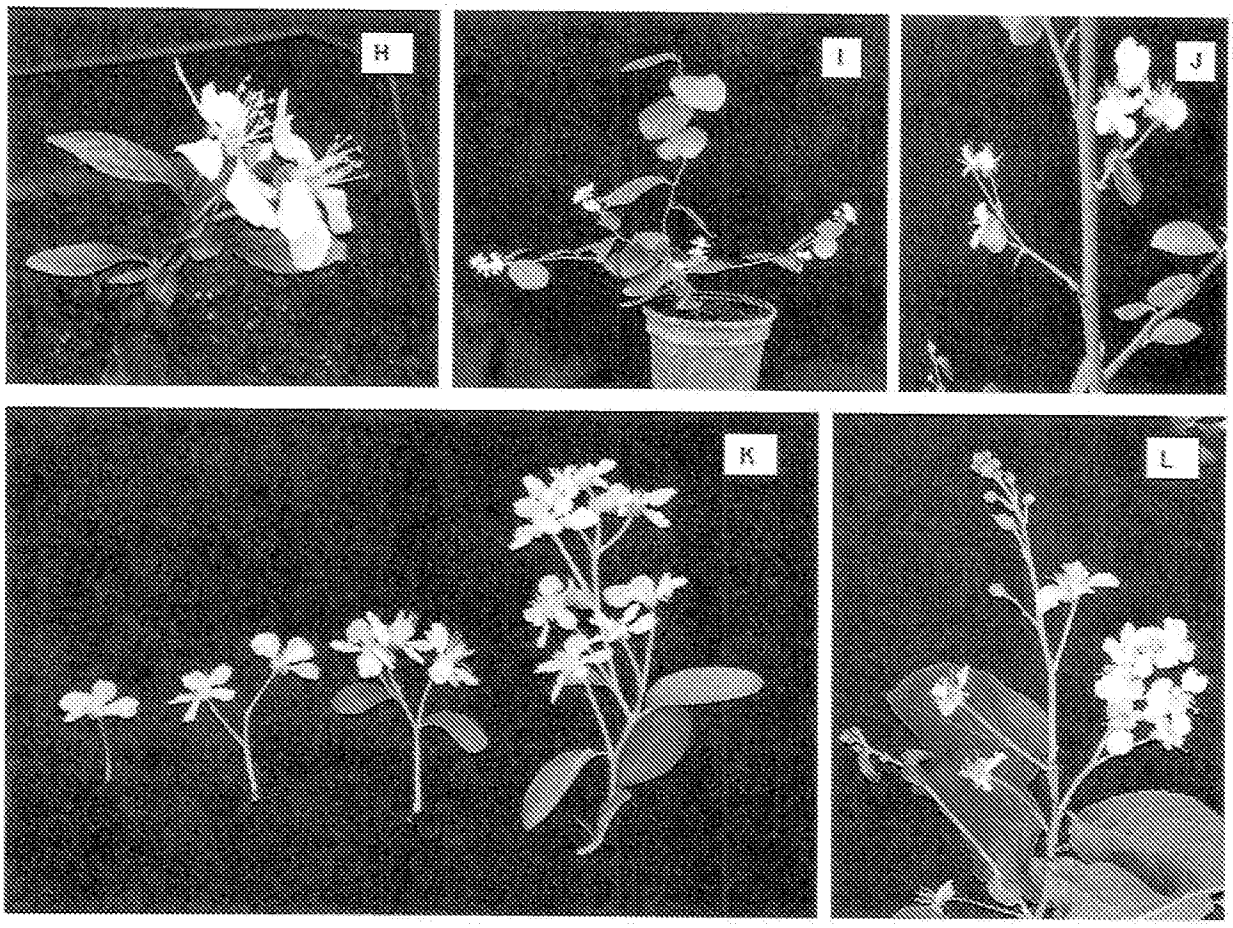


Fig. 2H-L

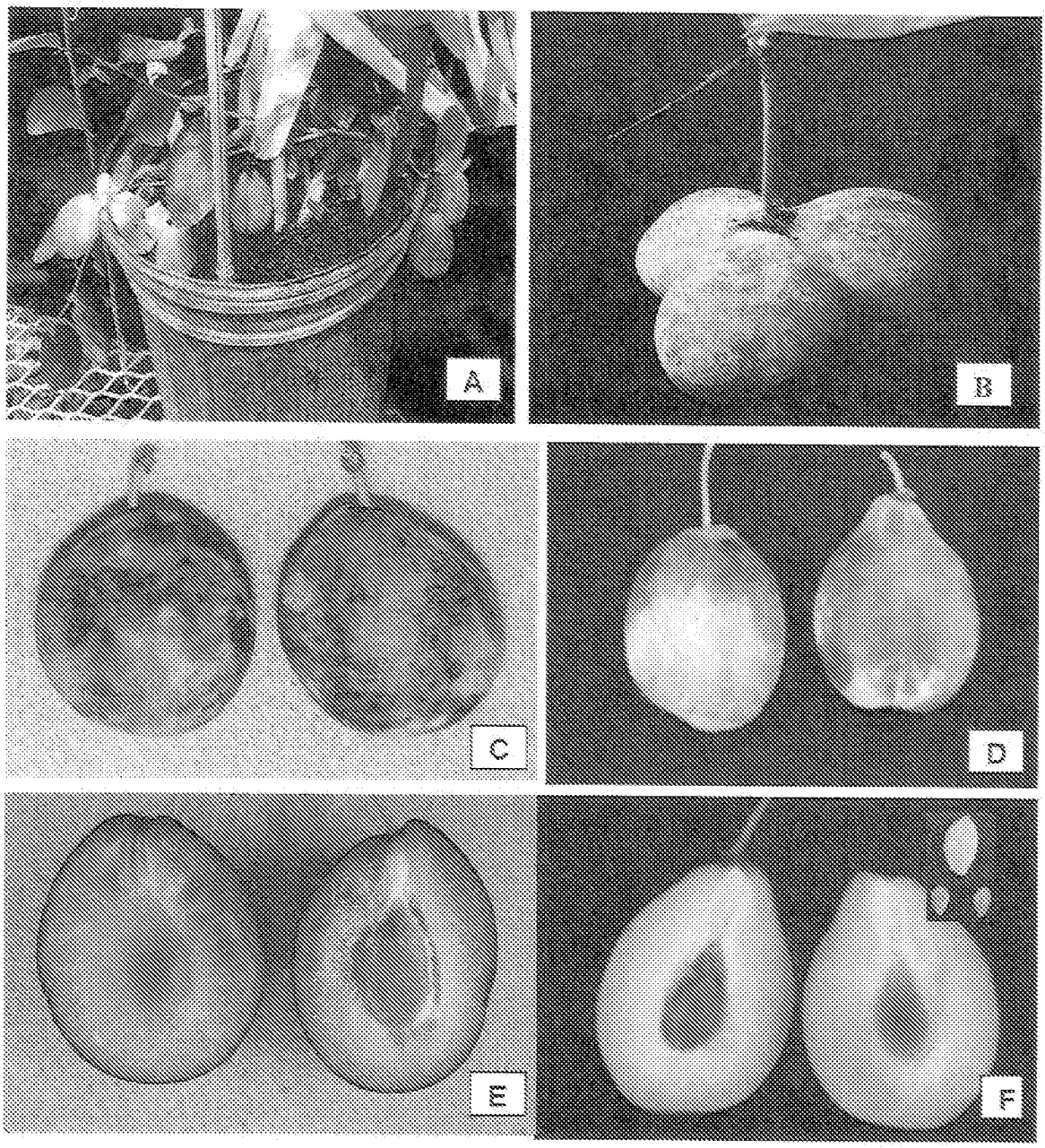


Fig. 3A-F

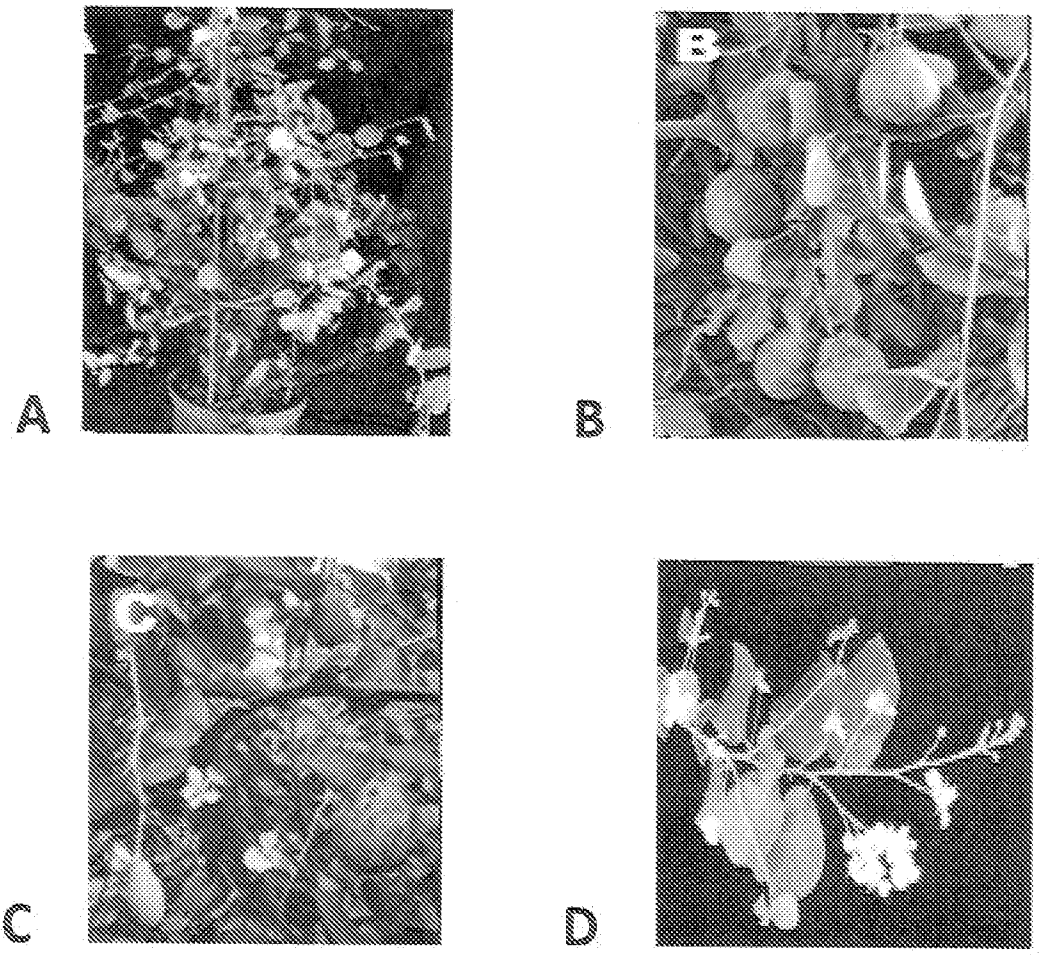


Fig. 4A-D

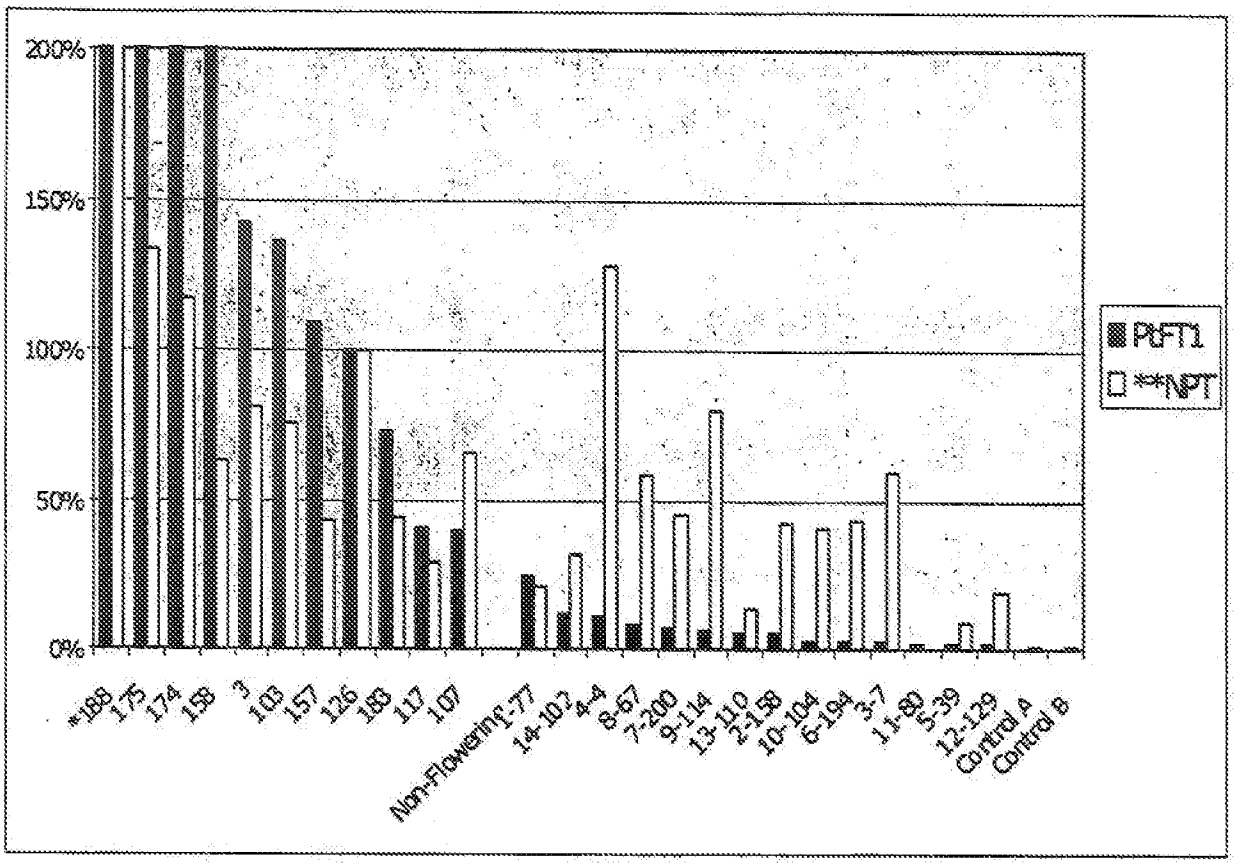

200%
150%
100%
50%
0%
PSFT1
**NPT
*188
175
174
158
3
103
157
126
153
117
107
Non-Flowering
7-77
14-107
4-4
8-67
7-200
9-114
13-110
2-158
10-104
6-194
3-7
11-80
5-39
12-129
Control A
Control B

Fig. 5

Fig. 6A-B

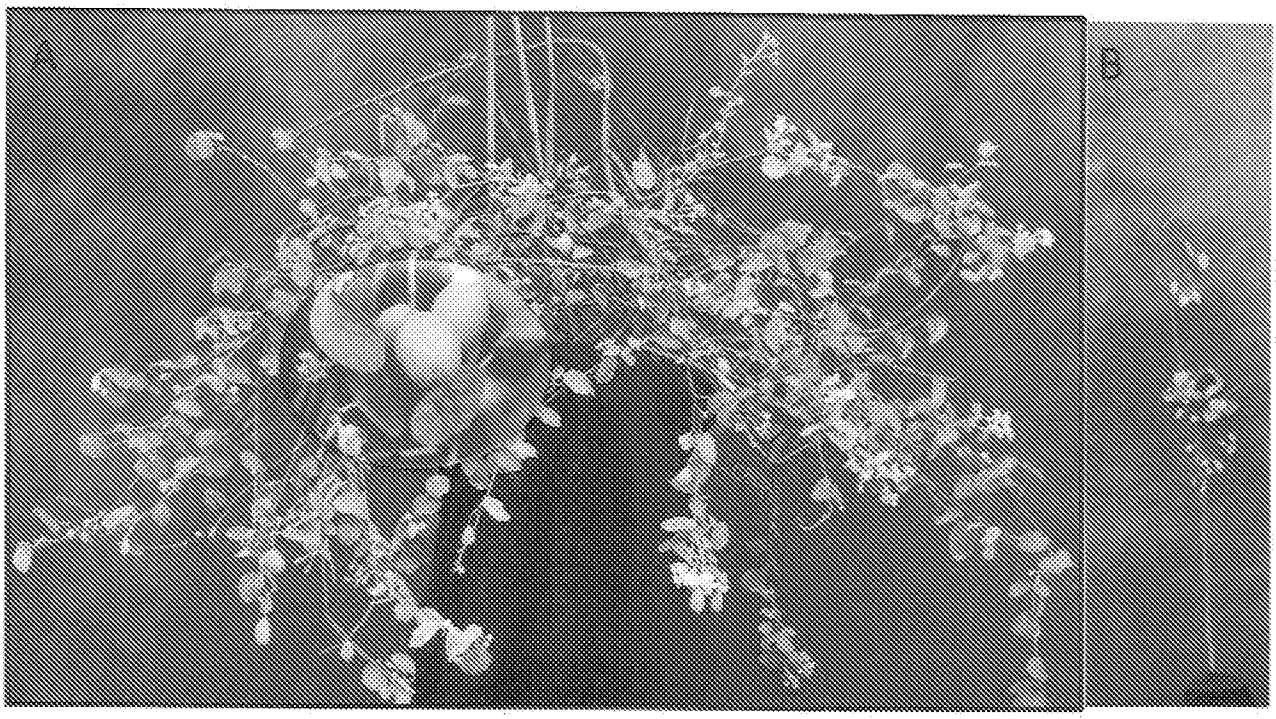
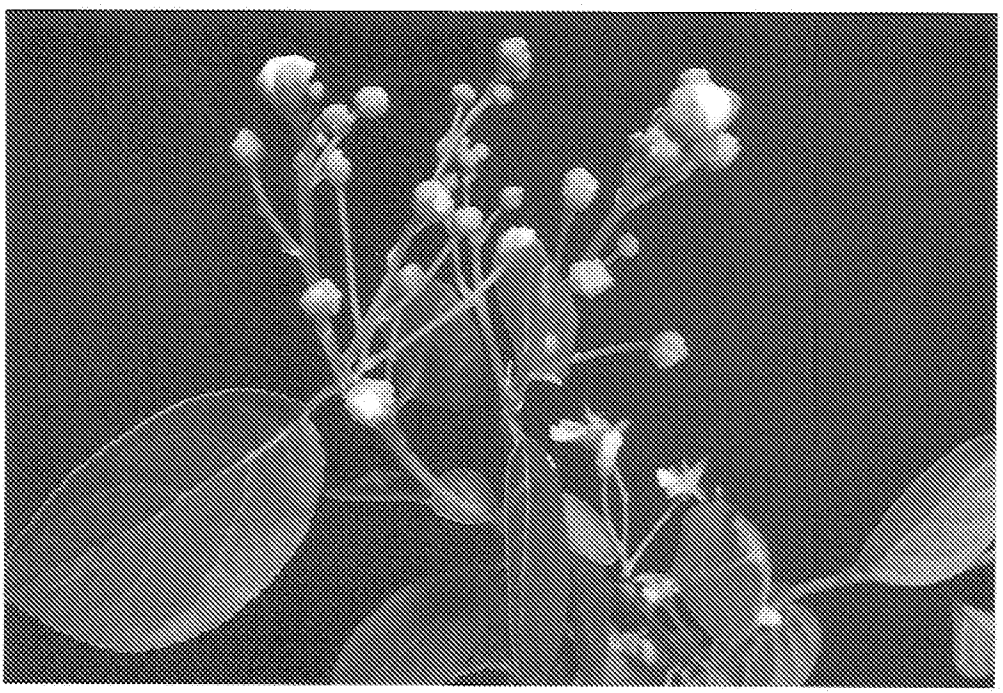


Fig. 7



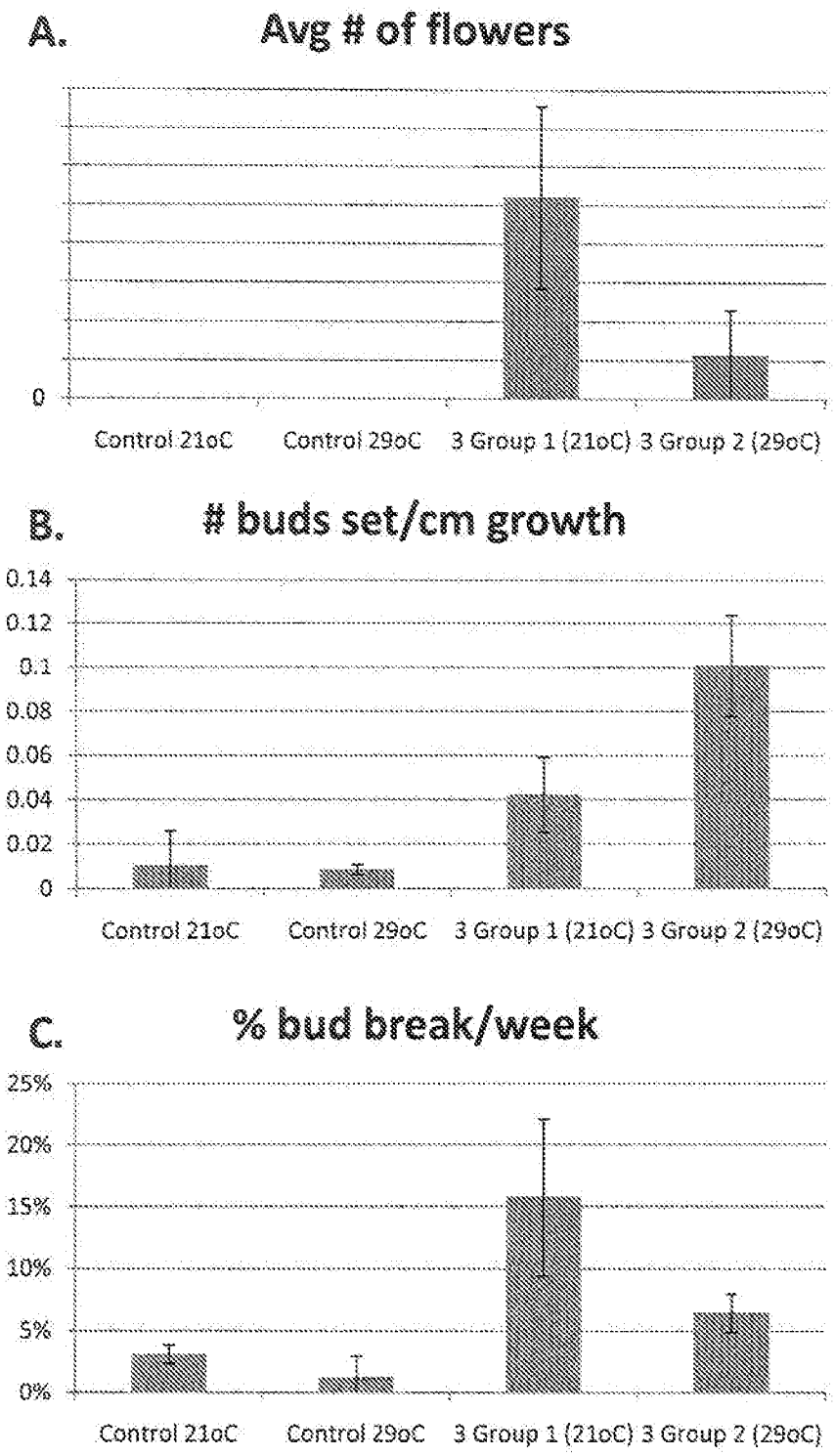

A.
Avg # of flowers
0
Control 21oC
Control 29oC
3 Group 1 (21oC)
3 Group 2 (29oC)
B.
buds set/cm growth
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Control 21oC
Control 29oC
3 Group 1 (21oC)
3 Group 2 (29oC)
C.
% bud break/week
25%
20%
15%
10%
5%
0%
Control 21oC
Control 29oC
3 Group 1 (21oC)
3 Group 2 (29oC)

Fig. 8

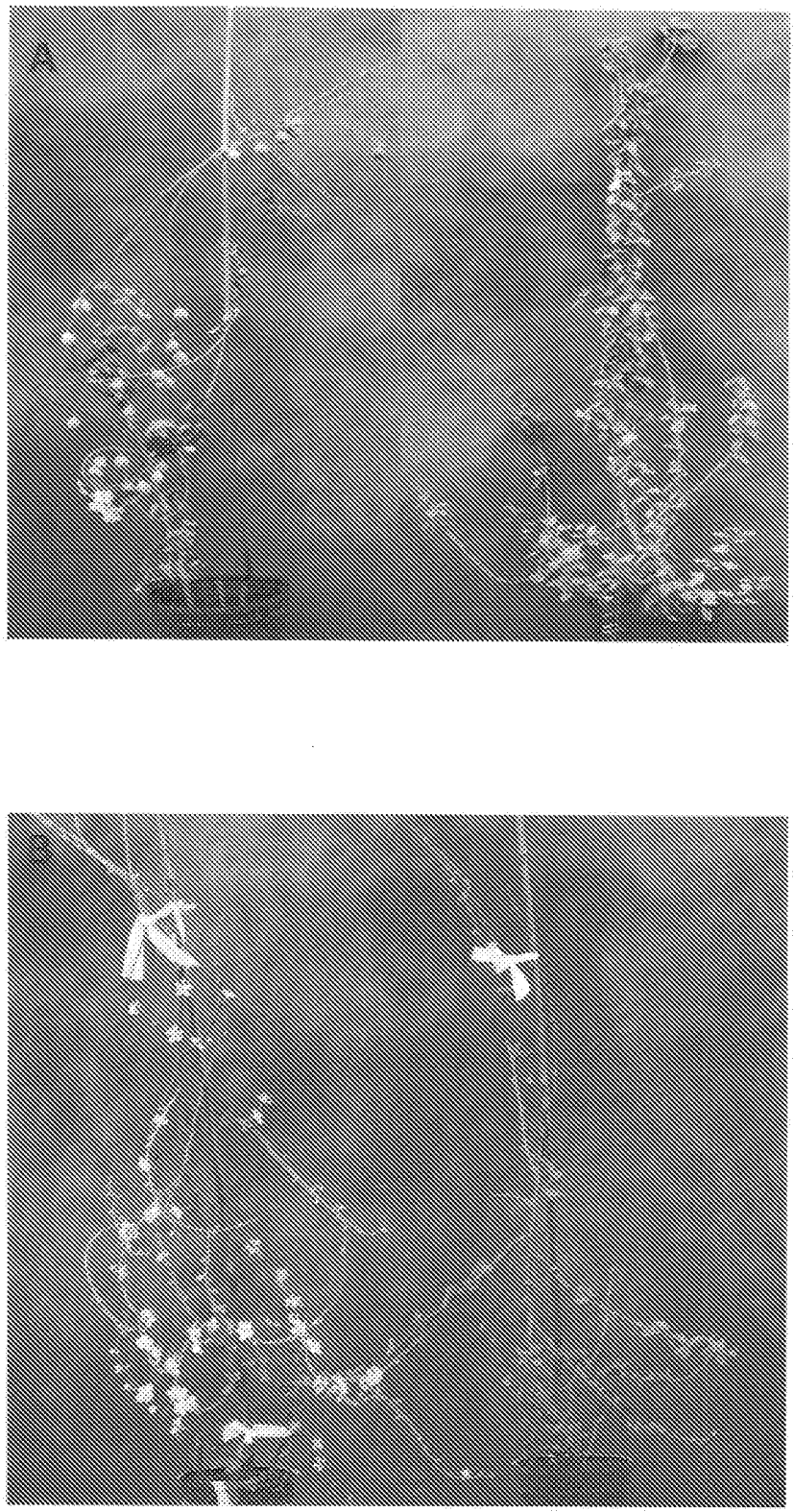


Fig. 9A-B

Generation Time (yr)
FasTrack
Standard
Insertion of Early Flowering (EF) transgene via plant transformation
<1
0
EF line
Introduce desired trait (R) into EF line by pollination
R
X
1
5
Disease Resistance (R) trait
EF line
Repeatedly backcross segregating EF lines w/ R trait to a high quality parent
R
1
5
R X
EF Progeny
High Quality Parent
R
1
5
Select non-EF plants (non-transgenic) with high quality traits + R trait and plant to field
<4yr
15yr
Improved Variety

Fig. 10

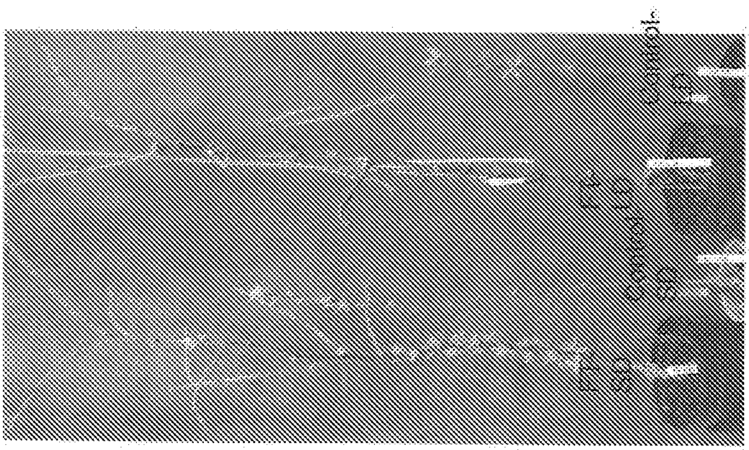


Fig. 11

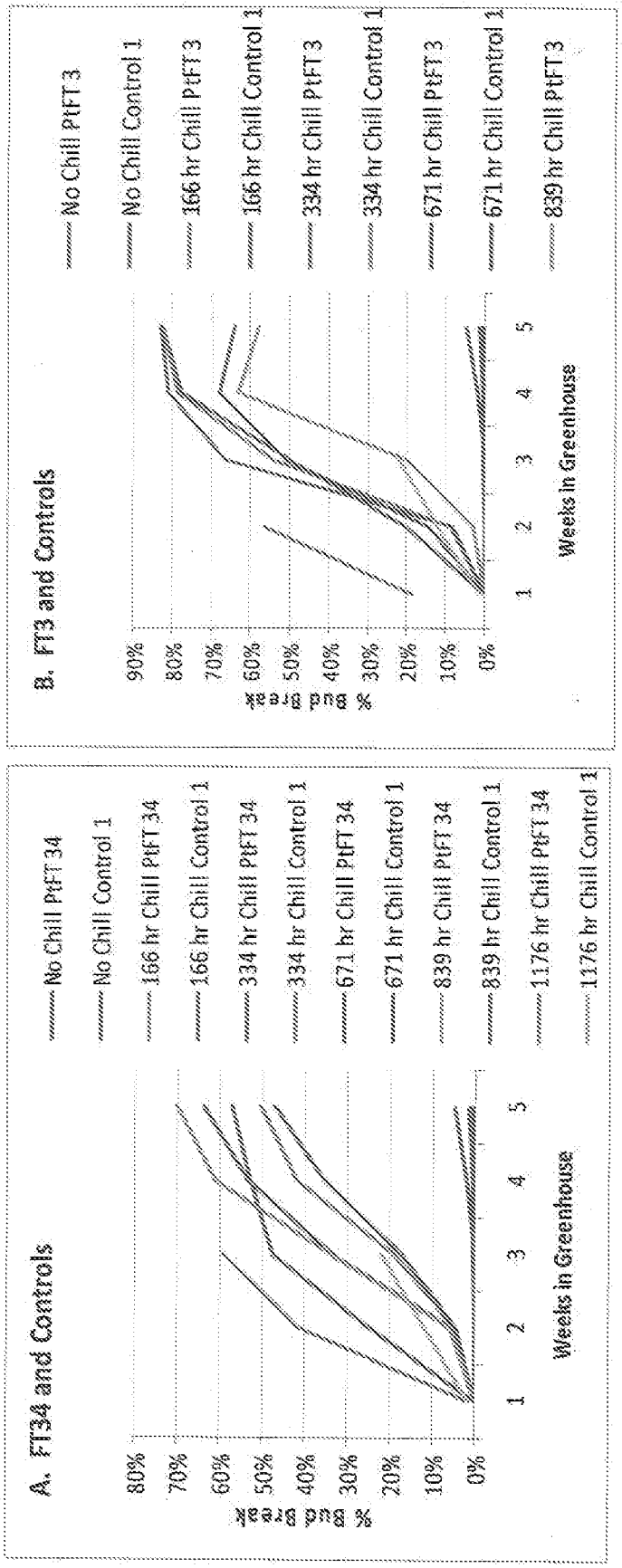

A. FT34 and Controls
% Bud Break
80%
70%
60%
50%
40%
30%
20%
10%
0%
1
2
3
4
5
Weeks in Greenhouse
No Chill PtFT 34
No Chill Control 1
166 hr Chill PtFT 34
166 hr Chill Control 1
334 hr Chill PtFT 34
334 hr Chill Control 1
671 hr Chill PtFT 34
671 hr Chill Control 1
839 hr Chill PtFT 34
839 hr Chill Control 1
1176 hr Chill PtFT 34
1176 hr Chill Control 1
B. FT3 and Controls
% Bud Break
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1
2
3
4
5
Weeks in Greenhouse
No Chill PtFT 3
No Chill Control 1
166 hr Chill PtFT 3
166 hr Chill Control 1
334 hr Chill PtFT 3
334 hr Chill Control 1
671 hr Chill PtFT 3
671 hr Chill Control 1
839 hr Chill PtFT 3

Fig. 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2010/058916

A. CLASSIFICATION OF SUBJECT MATTER

A01H 5/00(2006.01)i, C12N 15/82(2006.01)i, C12N 15/29(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A01H 5/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: Prunus domestica, populus trichocarpa flowering locuts T1, flower, gene, transgenic plum plant, fruit, et al.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GenBank accession no.BAD01576, GI: 38304185 (2004.06.08.) See the sequence	8
A	Plant Physiology, Vol.135: 201-211 (2004.05.) See the whole document; especiall materials and methods	8
A	Heredity, Vol.101: 351-358 (2008) See the whole document; especiall materials and methods	8
A	Tree Physiology, Vol.28: 1873-1882 (2008) See the whole document; especiall materials and methods	8
A	Int. J. Biometeorol, Vol.53: 287-298 (2009) See the whole document; especiall materials and methods	8
A	Plant Physiology and Biochemistry, Vol.47: 690-700 (2009) See the whole document; especiall materials and methods	8

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 JULY 2011 (28.07.2011)	**28 JULY 2011 (28.07.2011)**

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
Government Complex-Daejeon, 189 Cheongsa-ro,
Seo-gu, Daejeon 302-701, Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer
KIM Kee-Yeun
Telephone No. 82-42-481-8387

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2010/058916

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 23-26
because they relate to subject matter not required to be searched by this Authority, namely:

Claims 23-26 pertain to plant varieties, and thus relates to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1 (ii) of the Regulations under the PCT, to search.

2. ☒ Claims Nos.: 1-7,9-22
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claims 1-7, 9-22 are directed at or refer to PtFT1 cDNA are considered too broad and so inadequately supported by the description that no meaningful search could be made.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2010/058916

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
None			