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(71) Applicant: THE STATE OF ISRAEL, MINISTRY OF AGRICULTURE & RURAL DEVELOPMENT, AGRICULTURAL RESEARCH ORGANIZATION, (A.R.O.), VOLCANI CENTER [IL/IL]: P.O. Box 6, 5025001 Beit-Dagan (IL).

(72) Inventors: GRANOT, David; 4 Bustnai Street, 9210608 Jerusalem (IL). KELLY, Gilor; 20 HaZayit Street, 4680300 Beit-Elazari (IL). MOSHELION, Menachem; 16A HaMaApir Street, 7634816 Rechovot (IL).

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Abstract:
Methods of modulating stomata conductance and plant expression constructs for executing same are provided. According to an embodiment, the plant expression construct comprises a nucleic acid sequence encoding a hexokinase under a transcriptional control of a guard cell-specific cis-acting regulatory element. Also provided are methods of using the constructs and transgenic plants, plant cells and plant parts expressing same.

Title:
METHODS OF MODULATING STOMATA CONDUCTANCE AND PLANT EXPRESSION CONSTRUCTS FOR EXECUTING SAME

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(54) Title: METHODS OF MODULATING STOMATA CONDUCTANCE AND PLANT EXPRESSION CONSTRUCTS FOR EXECUTING SAME

(57) Abstract: Plant expression construct are provided. According to an embodiment, the plant expression construct comprises a nucleic acid sequence encoding a hexokinase under a transcriptional control of a guard cell-specific cis-acting regulatory element. Also provided are methods of using the constructs and transgenic plants, plant cells and plant parts expressing same.
METHODS OF MODULATING STOMATA CONDUCTANCE AND PLANT
EXPRESSION CONSTRUCTS FOR EXECUTING SAME

This application claims the benefit of priority under 35 USC 119(e) of U.S. Provisional Patent Application No. 61/569,251 filed December 11, 2011, the contents of which are incorporated herein by reference in their entirety.

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to methods of modulating stomata conductance and plant expression constructs for executing same.

Stomata are dynamic pores in the impermeable protective cuticle that coats the aerial parts of land plants, which evolved primarily to save water. Stomata, which are comprised of two guard cells and the pore they circumscribe, open at dawn to allow the entry of atmospheric carbon dioxide (CO$_2$) for photosynthesis, at the cost of extensive transpirational water loss. The stomata close when carbon fixation and utilization are less efficient, in order to reduce the loss of water via transpiration (Assmann, 1993). At the mechanistic level, stomata open in response to increases in the osmolality of the guard cells. These increases in osmolality are followed by the movement of water into the guard cells, which increases their volume and opens the stomata (Taiz and Zeiger, 1998). Stomatal closure occurs in the opposite manner; as the osmolality of guard cells is reduced, their volume decreases.

Water scarcity is a serious problem that will be exacerbated by global climate change. Abiotic stresses, especially drought and increased salinity, are primary causes of crop loss worldwide. Moreover, agriculture currently uses over 70% (86% in developing countries) of available freshwater. One of the approaches that may be adopted to save water in agriculture is the development of plants that use less water yet maintain high yields in conditions of water scarcity. As plants lose over 95% of their water via transpiration through stomata, the engineering of stomatal activity is a promising approach to reduce the water requirement of crops and to enhance productivity under stress conditions.

Additional background art includes


**SUMMARY OF THE INVENTION**

According to an aspect of some embodiments of the present invention there is provided a plant expression construct comprising a nucleic acid sequence encoding a hexokinase under a transcriptional control of a guard cell-specific cis-acting regulatory element.

According to an aspect of some embodiments of the present invention there is provided a plant expression construct comprising a nucleic acid sequence encoding a nucleic acid agent for silencing expression of a hexokinase, wherein expression of the nucleic acid agent is under a transcriptional control of a guard cell-specific cis-acting regulatory element.

According to some embodiments of the invention, the guard cell-specific cis-acting regulatory element is inducible.

According to some embodiments of the invention, the guard cell-specific cis-acting regulatory element is constitutive.

According to some embodiments of the invention, the guard cell-specific cis-acting regulatory element is a guard-cell specific promoter.

According to some embodiments of the invention, the guard-cell specific promoter is KST1 promoter.

According to an aspect of some embodiments of the present invention there is provided a method of regulating plant stomata conductance, the method comprising modulating in the plant the level and/or activity of a hexokinase in a guard cell specific manner, thereby regulating plant conductance.
According to some embodiments of the invention, the modulating is upregulating.

According to some embodiments of the invention, the upregulating is effected by introducing the nucleic acid construct of claim 1 into the plant.

According to some embodiments of the invention, the modulating is downregulating.

According to some embodiments of the invention, the downregulating is effected by introducing into the plant a nucleic acid silencing agent under a transcriptional control of a guard cell-specific cis-acting regulatory element.

According to an aspect of some embodiments of the present invention there is provided a method of decreasing plant stomata conductance, the method comprising introducing into a cell of a plant the nucleic acid construct, thereby decreasing the stomata conductance of the plant.

According to an aspect of some embodiments of the present invention there is provided a method of increasing water use efficiency of a plant, the method comprising introducing into a cell of the plant the nucleic acid construct, thereby increasing water use efficiency of the plant.

According to an aspect of some embodiments of the present invention there is provided a method of increasing tolerance of a plant to drought, salinity or temperature stress, the method comprising introducing into a cell of the plant the nucleic acid construct, thereby increasing tolerance of the plant to drought, salinity or temperature stress.

According to an aspect of some embodiments of the present invention there is provided a method of increasing biomass, vigor or yield of a plant, the method comprising introducing into a cell of the plant the nucleic acid construct, thereby increasing the biomass, vigor or yield of the plant.

According to an aspect of some embodiments of the present invention there is provided a method of increasing tolerance of a plant to biotic stress, the method comprising introducing into a cell of the plant the nucleic acid construct, thereby increasing tolerance of the plant to biotic stress.

According to an aspect of some embodiments of the present invention there is provided a transgenic plant or a part thereof comprising the plant expression construct.
According to an aspect of some embodiments of the present invention there is provided an isolated plant cell or a plant cell culture comprising the plant expression construct.

According to some embodiments of the invention, the part of the transgenic plant is a seed.

According to some embodiments of the invention, the part of the transgenic plant is a leaf.

According to some embodiments of the invention, the seed is a hybrid seed.

According to some embodiments of the invention, the method further comprises growing the plant under water deficient conditions.

According to some embodiments of the invention, the method further comprises growing the plant under salinity.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIGS. 1A-C are graphs showing that sucrose stimulates stomatal closure via hexokinase. FIG. 1A - Representative light microscopy images of stomata taken from epidermal imprints 3 h after treatment with 100 mM sorbitol or 100 mM sucrose (white bar = 20 µm). B, Stomatal response to sucrose in wild-type (WT) and *AtHXK1*-
expressing plants (HK4) was assayed with intact leaves immersed for 3 h in artificial apoplastic sap (Wilkinson and Davies, 1997) containing 100 mM sorbitol (as an osmotic control), 100 mM Suc or 100 mM sucrose together with 20 mM of the hexokinase inhibitor N-acetyl-glucoseamine (NAG). Epidermal imprints were then taken and stomatal aperture was measured. C, The stomatal responses of WT plants to the different sugar combinations were assayed as described in (FIG. IB), with 200 mM mannitol serving as an additional osmotic control. The data shown in FIGs. IB, C are means of 300 stomata from six independent biological repeats ± SE. Different letters indicate a significant difference (t test, $P < 0.05$).

FIGs. 2A-D show that elevated expression of hexokinase enhances stomatal closure and decreases transpiration. Stomatal aperture (FIG. 2A) and stomatal conductance (FIG. 2B) were determined for control (WT) and transgenic plants expressing different levels of $AtHXK1$ (HK38 > HK4 > HK37) (Dai et al, 1999). Aperture data are means of 200 stomata from four independent repeats ± SE. Stomatal conductance data are means of six independent repeats ± SE. Different letters indicate a significant difference (t test, $P < 0.05$). FIG. 2C - The rate of transpiration normalized to the total leaf area was monitored simultaneously and continuously throughout the day and the data are given as the means ± SE for each 10th sampling point ($n = 6$). FIG. 2D - A negative correlation was observed between whole-plant relative daily transpiration and relative hexokinase-phosphorylation activity. The transpiration data were normalized to the total leaf area and the amount of water taken up by the neighboring submerged fixed-size wick each day, which was set to 100%. WT hexokinase activity was set to 100%.

FIGs. 3A-E show that $AtHXK1$ reduces transpiration primarily when expressed in leaves. Reciprocal grafting (FIG. 3A) and triple-grafting (FIG. 3D) procedures were performed at the seedling stage and plants were photographed and used for transpiration measurements about 4 weeks after grafting. The yellow arrows and brackets indicate the location of the grafts. FIG. 3B - Whole-plant relative daily transpiration of reciprocal-grafted plants. Data were normalized to the total leaf area and the amount of water taken up by the neighboring submerged fixed-size wick each day, which was set to 100%. Data are given as means of four independent repeats ± SE. Different letters indicate a significant difference (t test, $P < 0.05$). FIG. 3C - Transpiration rate normalized to the
total leaf area of reciprocal-grafted plants was monitored simultaneously and continuously throughout the day. The data are given as the means ± SE for each 10th sampling point (n = 4). FIG. 3E - Relative daily transpiration of whole triple-grafted plants calculated as in (FIG. 3B).

FIGs. 4A-B are graphs showing that suppression of HXK inhibits stomatal closure in response to Sue. FIG. 4A - Quantitative measurements of the real-time expression of tomato LeHXKl-3 genes in wild-type tomato (WT) and in two independent tomato lines with antisense suppression of HXK, aHK1 and aHK2. Data are means of three independent biological repeats ± SE. Asterisks denote significant differences relative to the WT (t test, P < 0.05). FIG. 4B - Stomatal response to Sue in WT, two antisense (aHK1 and aHK2) and AtHXKl-expressing (HK4) lines was assayed in intact leaves that were immersed in artificial apoplastic sap (Wilkinson and Davies, 1997) containing 100 mM Sue for 3 h. Data are given as means of 400 stomata from eight independent biological repeats ± SE. Different letters indicate a significant difference (t test, P < 0.05).

FIG. 5 is a graph showing that glucose (Glc) and sugars that can be phosphorylated, but not metabolized, stimulate stomatal closure. Stomatal responses to different sugars were assayed in intact leaves of wild-type plants. The leaves were immersed for 3 h in artificial apoplastic sap (Wilkinson and Davies, 1997) containing mannitol (as an osmotic control), Glc, 2-deoxyglucose (2-dG) or mannose. Epidermal imprints were then taken and stomatal aperture was measured. Data are given as means of 400 stomata from eight independent biological repeats ± SE. Different letters indicate a significant difference (t test, P < 0.05).

FIGs. 6A-F show that Sue stimulates ABA-dependent NO production in guard cells that is mediated by HXK. FIGs. 6A-B - Nitric oxide (NO) levels were monitored in guard cells from epidermal peels of wild-type (WT) and AtHXKl-expressing (HK4) plants using the fluorescent NO indicator dye DAF-2DA. Relative fluorescence levels of guard cells (white bars) and stomatal apertures (black bars) were determined after 30 min of treatment with MES buffer (control) or MES containing either 100 mM Sue or 100 mM sorbitol as an osmotic control. Representative fluorescence images are shown above the fluorescence columns (bar = 10 μm). Data are given as means ± SE of 90 stomata (FIG. 6A) or 60 stomata (FIG. 6B) for each treatment with three to four
independent biological repeats of each treatment. FIG. 6C - Relative fluorescence levels of WT guard cells were determined after 30 min of treatment with MES buffer (control), MES containing 20 mM of the hexokinase inhibitor N-acetyl-glucoseamine (NAG), or 100 mM Sue with or without 20 mM NAG. Representative fluorescence images are shown above the fluorescence columns (bar = 10 µm). Data are given as means of 60 stomata from three independent biological repeats per treatment ± SE. FIG. 6D - Confocal images of NO production in guard cells of epidermal peels treated with 20 mM NAG only (left), 30 min after the addition of 100 mM Sue (middle) and 30 min after the NAG was washed out with 100 mM Sue (right). The assay was conducted as the same epidermal strip was being photographed (bar = 20 µm). FIG. 6E - Relative fluorescence levels of guard cells from an epidermal strip treated as in (FIG. 6D). Data are given as means of 40-60 stomata ± SE. FIG. 6F - Confocal images of NO production in guard cells of epidermal peels of Sitiens (ABA-deficient mutants) after 30 min of treatment with MES buffer containing either 100 mM Sue (left) or 100 µM ABA (right); bar = 10 µm. Different lower-case letters in (FIGs. 6A-C, E) indicate a significant difference among the treatments with respect to the fluorescence data and different upper-case letters in (FIG. 6A) indicate a significant difference among the treatments with respect to the stomatal aperture data (t test, P < 0.05).

FIGs. 7A-E show that GFP expression under the control of the KST1 promoter is specific to guard cells. FIG. 7A- Confocal images of wild-type (WT) (panels 1-4) and transgenic tomato leaves (panels 5-8) of plants with guard-cell specific expression of GFP (designated GCGFP) under the control of the KST1 promoter. Panels 1 and 5 are images of GFP fluorescence (stained green), panels 2 and 6 are chlorophyll autofluorescence (stained magenta), panels 3 and 7 are white light images and panels 4 and 8 are merged images. B-E, Confocal images of WT (left) and transgenic Arabidopsis GCGFP plants (right). Images were taken from leaves (FIGs. 7B and C, bars = 50 µm and 5 µm, respectively), hypocotyls (FIG. 7D, bar = 100 µm) and roots (FIG. 7E, bar = 50 µm). All panels are merged images of white light, chlorophyll autofluorescence (magenta) and GFP fluorescence (green).

FIGs. 8A-F show that guard cell-specific expression of AtHXKL induces stomatal closure and reduces transpiration of tomato and Arabidopsis plants. FIG. 8A - Representative images of wild-type (WT) and two independent transgenic tomato lines
expressing \textit{AtHXK1} specifically in guard cells (GCHXK7 and 12). FIGs. 8B and C - Stomatal conductance ($g_s$) and whole-plant relative daily transpiration of WT and two independent transgenic tomato lines (GCHXK7 and 12). Stomatal conductance data are given as means of four independent repeats ± SE. Transpiration data were normalized to the total leaf area and the amount of water taken up by the neighboring submerged fixed-size wick each day, which was set to 100%. Data from three consecutive days are presented. Data for each day are given as means of four independent repeats ± SE. FIG. 8D - Representative images of WT Arabidopsis (Col. ecotype) and two independent transgenic lines expressing \textit{AtHXK1} specifically in guard cells (GCHXK1 and 2). FIGs. 8E and F - Stomatal conductance and transpiration measurements of WT, two independent transgenic Arabidopsis lines, GCHXK1 and GCHXK2 (Col. ecotype), and of the \textit{gin} 2-I (\textit{AtHXK1} null mutant, \textit{her}. ecotype). Arrows indicate increased or decreased conductance and transpiration relative to the WT. Data are given as means (± SE) of 8 and 12 independent repeats for the GCHXK and \textit{gin}2-I lines, respectively. Asterisks denote significant differences relative to the WT ($t$ test, $P < 0.05$).

FIG. 9 shows that GFP expression under the control of the \textit{FBPase} promoter is specific to mesophyll cells. Confocal images of transgenic tomato and Arabidopsis leaves of plants with mesophyll specific expression of GFP (designated MCGFP) under the control of the \textit{FBPase} promoter. Images are merge of GFP fluorescence (stained green) and white light images (bar = 100 μm). Fluorescence is specific to mesophyll cells.

FIGs. 10A-D are graphs showing that elevated expression of hexokinase in guard cells reduces transpiration while photosynthesis remains unchanged, thus improving instantaneous water use efficiency. Gas exchange analysis of GCHXK and WT plants was assayed using a Li-6400 portable gas-exchange system (LI-COR), stomatal conductance (FIG. 10A), transpiration (FIG. 10B), photosynthesis (FIG. IOC) and instantaneous water use efficiency (TWUE, FIG. 10D) were measured and calculated under favorable growth conditions. Data are mean ± SE (n=10 for WT and n=20 for 10 different transgenic lines, two measurements each). Star denotes significant difference ($t$ test, $P < 0.05$).

FIGs. 11A-C show that elevated expression of hexokinase in guard cells reduces whole plant transpiration and increases water use efficiency. FIGs. 11A-B - Whole plant
relative daily transpiration (RDT) was analyzed using the large-scale lysimeter system as described in Example 1. WT and two GCHXK transgenic lines (GCHXK7, GCHXK12) were put on scales. Transpiration and total plant weight were documented every 3 minutes during the experiment in which plants were grown under normal conditions for 10 days, than subjected to drought stress for 3 days, followed by recovery irrigation process for additional 7 days. Data were normalized to the total plant weight and the amount taken up by the neighboring submerged fixed-size wick each day, which was set to 100% and served as a reference for the temporal variations in the potential transpiration. FIG. 11A - Day by day Relative daily transpiration during the whole experiment. Data are means of four independent repeats ± SEM. FIG. 11B - Relative daily transpiration of selected days in each treatment. Data are means of four independent repeats ± SEM; Star denotes significant difference (t test, \(P < 0.05\)). FIG. 11C - Water use efficiency was calculated by the ratio between plant weight accumulation and plant water loss, each day per each plant. Data are means of four independent repeats ± SEM; Star denotes significant difference (t test, \(P < 0.05\)). (A-magnified) RDT of WT and GCHXK plants during the shift from normal irrigation (day 10) to drought conditions (day 11). Red and green arrows indicate RDT decline (represented by slope) of WT and GCHXK respectively after plants were exposed to drought.

FIGs. 12A-F show that elevated expression of hexokinase in guard cells reduces transpiration rate and stomatal conductance throughout the day, while displaying normal growth. Whole plant relative transpiration rate (FIG. 12A) and stomatal conductance \(g_s\) (FIG. 12B) were analyzed using the large-scale lysimeter system as described in methods. WT and two GCHXK transgenic lines were put on scales. Transpiration rate, \(g_s\), light intensity (FIG. 12E), vapor pressure deficit (VPD, FIG. 12F) were simultaneously documented every 3 minutes during the experiment in which plants were grown under normal conditions. Data for FIGs. 12A and B were normalized to the total leaf area and the amount taken up by the neighboring submerged fixed-size wick each day, which was set to 100% and served as a reference for the temporal variations in the potential transpiration. FIG. 12C - Total plant leaf area, FIG. 12D - Total plant weight.
FIG. 13 show transpiration rate of WT and GCHXK plants under drought conditions. Whole plant transpiration rate was analyzed using the large-scale lysimeter system as described in Example 1. WT (blue) and GCHXK transgenic lines (green) were put on scales. Transpiration rates were documented for 9 days after exposing the plants to gradually increased - drought conditions, by fully stopping the irrigation. The rate of transpiration normalized to the total leaf area was monitored simultaneously and continuously throughout the day and the data are given as the means ± SE for each sampling point. Data were normalized to the total leaf area and the amount taken up by the neighboring submerged fixed-size wick each day, which was set to 100% and served as a reference for the temporal variations in the potential transpiration. Star denotes the day in which transpiration transition between WT and GCHXK had occurred.

FIGs. 14A-B show the yield production of transgenic plants expressing hexokinase specifically in guard cells. FIG. 14A - Number of fruits collected from WT and GCHXK plants (4 independent lines). FIG. 14B - Representative images of wild-type (WT) and transgenic tomato plant expressing AtHXK1 specifically in guard cells (GCHXK7).

FIGs. 15A-C show the yield production of transgenic plants expressing hexokinase specifically in guard cells, under limited water-supply conditions. FIG. 15A - Plants were grown under controlled commercial greenhouse conditions, following expert instructions with regard to growing procedures (Soil system, irrigation, fertilization etc.). Seedlings were planted in a mixed up order threw out the entire planting-row and the same order was kept in each row. Each row was irrigated differentially; either fully (100%) or partially (75%, 50% and 25% irrigation regimes). Since the initial fruit breaker stage, fruits were collected, counted and weighted for each individual plant for 4 weeks time. Cumulative fruit weight (FIG. 15B) and fruit number (FIG. 15C) of WT (blue) and GCHXK (green) plants were than averaged for each irrigation regime. Blue and green arrows indicates decreased fruit weight of WT and GCHXK plants respectively when shifting from 75% to 50% irrigation.

FIGs. 16A-F show that guard cell-specific expression of AtHXK1 induces stomatal closure, reduces transpiration and increases leaf temperature without lowering photosynthesis or mesophyll conductance for CO₂, thus enhances water use efficiency of Arabidopsis plants. Stomatal conductance (FIG. 16A), transpiration (FIG. 16B),
photosynthesis (FIG. 16C) and mesophyll conductance for \( \text{CO}_2 \) (gm, FIG. 16D) measurements of WT and transgenic Arabidopsis plants expressing \textit{AtHXK}I specifically in guard cells (GCHXK). FIG. 16E - instantaneous water use efficiency (IWUE) of WT and GCHXK plants. FIG. 16F - Leaf temperatures (warmer leaves - stomatal closure) of WT and GCHXK plants were determined using ThermaCam researcher pro 2.10 software. Data points are the means ± SE from 6 biological repeats in FIGs. 16A-E and of 12 biological repeats in FIG. 16F. An asterisk denotes a significant difference relative to the wild type (t test, \( P < 0.05 \)).

FIGs. 17A-B are schematic maps of binary vector pGreen0029 containing KST1 promoter, \textit{AtHXK}I cDNA (FIG. 17A) or GFP (FIG. 17B) and a terminator: Vector also contains nos-Kan and neomycin phosphotransferase II (\textit{Npt}II) genes as selectable markers for bacteria and plant transformation.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to methods of modulating stomata conductance and plant expression constructs for executing same.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or earned out in various ways.

Water is the major factor limiting the growth and development of many land plants. Stomata, composed of two guard cells, are the chief gates controlling plants' water loss. Many environmental and physiological stimuli control stomatal opening, but they all function through the regulation of guard-cell osmolality. Increased guard-cell osmolality leads to the opening of the stomata and decreased osmolality causes the stomata to close. The prevailing paradigm is that sucrose acts as an osmoticum in the guard cells, thereby contributing to the opening of the stomata.

While conceiving the present invention, the present inventors have found that contrary to the prevailing paradigm, sucrose closes stomata via a non-osmotic mechanism (see Example 2). Furthermore, the guard cells' response to sucrose is dependent on the sugar-sensing enzyme hexokinase (HXK), which triggers the abscisic acid-signaling pathway within the guard cells, leading to stomatal closure.
Thus, while reducing the present invention to practice, the present inventors have found that modulation of hexokinase activity or expression correlates with stomatal aperture.

As is illustrated herein below and in the Examples section which follows, the present inventors have overexpressed HXK in the stomata of tomato plants (in a guard-cell specific manner). Surprisingly, while photosynthesis remained unchanged (Figure 10C), stomatal conductance (indicating stomatal aperture, Figure 10B) and transpiration (Figure 10A) were reduced. Similar results were obtained while monitoring the same parameters all day long (Figures 12A-D). Importantly, by measuring total plant leaf area and weight (Figures 12C and 12D respectively), the present inventors discovered that even though plants have consumed less water (Figure 12A), growth was not impaired, and was even improved. Saving water without affecting plant growth improves whole plant water use efficiency. Elevated expression of hexokinase in guard cells improves yield production (Figures 14A-B) even under limited water supply (Figures 15A-C). Similar results were observed in Arabidopsis. These results demonstrate that the same transgenic insertion of hexokinase under guard-cell specific promoter used in the case of Tomato (Solanaceae family) is universally applicable while affecting stomata and increases water use efficiency in the case of Arabidopsis (Brassicaceae family) as well, and that this technique could be implemented in other species as well.

Unlike previous studies, which relied on correlations between sucrose content and stomatal aperture, this study took a functional approach to the examination of the effects of sucrose and its cleavage products on stomatal behavior. It is now proven that sucrose stimulates a guard cell-specific response that is mediated by HXK and ABA and leads to stomatal closure. Without being bound to theory it is suggested that this response presents a natural feedback mechanism aimed at reducing transpiration and conserving water under excess of photosynthesis, thus coordinating between photosynthesis and transpiration.

Thus, according to an aspect of the invention there is provided a method of regulating plant stomata conductance, the method comprising modulating in the plant the level and/or activity of a hexokinase in a guard cell specific manner, thereby regulating stomata conductance and plant transpiration.
As used herein the phrase "stomata conductance" refers to gaseous exchange through the stomata pore complex. Stomata conductance is regulated by stomata aperture. Stomatal conductance affects plant transpiration and therefore the present methodology according to this aspect of the invention also regulated plant transpiration.

As used herein the phrase "regulating plant stomata conductance" refers to increase or decrease in stomata conductance. The increase or decrease may be by at least 2%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or more say 90% or 100% (e.g., 20-40%).

As used herein the term "hexokinase", abbreviated as HKK, and referred to herein as "the transgene" or "the polypeptide", refers to the enzyme that typically phosphorylates hexoses (six-carbon sugars), forming hexose phosphate and having the E.C. Number 2.7.1.1. HKK as used herein also refers to hexokinase-like (HKL) protein that binds hexose and transmits a signal independent of its kinase (hexose phosphorylation) activity.

Hexokinases according to the present teachings may be about 100 kD in size as of most multicellular organisms (e.g., mammalian and plants). They consist of two halves (N and C terminal), which share much sequence homology. This suggests an evolutionary origin by duplication and fusion of a 50kD ancestral hexokinase similar.

The hexokinase may be naturally occurring or may comprise/consist of a synthetic sequence (i.e., man-made) as long as it retains a hexokinase activity.

Due to their high conservation level, the hexokinase of the present invention can be of a plant- or an animal origin. According to a specific embodiment, the hexokinase is a plant hexokinase.

The hexokinases can be categorized according to their cellular localization. Thus, the HKKs may be associated with the mitochondria, associated with or within plastids or present in the cytosol. To date, all of the HKKs examined in eudicots have been found to have either a plastidic signal peptide (type A) or an N-terminal membrane anchor domain (type B), however, cytosolic hexokinases are also contemplated for use according to the present teachings. According to a specific embodiment, the hexokinase is a type B (mitochondrial associated) HKK.

As used herein "a hexokinase activity" refers to the ability of the enzyme to regulate stomata conductance. The enzyme may bind hexose and stimulate the abscisic
acid (ABA) pathway which controls stomata conductance. The activity may be kinase independent.

Non limiting examples of hexokinases which are contemplated according to the present teachings are provided in Table 1 herein below.

<table>
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<tr>
<th>Species</th>
<th>Gene</th>
<th>Accession</th>
<th>Type/Physiological Function</th>
<th>References</th>
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<tr>
<td>AtHXK1</td>
<td>AT4G29</td>
<td>AT4G29 130</td>
<td>Type B M, N</td>
<td>Glc sensing PGD Mediates sugar and hormonal interactions Growth and development Photosynthetic gene repression Transpiration Actin filament reorganization Oxidative stress response Pathogen resistance Directional root growth Leaf senescence (Jang et al., 1997; Dai et al., 1999; Yanagisawa et al., 2003; Moore et al., 2003; Leon and Sheen, 2003; Kim et al., 2006; Pourtaii et al., 2006; Cho et al., 2006a; Rolland et al., 2006; Chen, 2007; Aki et al., 2007; Balasubramanian et al., 2007, 2008; Sarowar et al., 2008; Karve et al., 2008; Ju et al., 2009; Karve et al., 2010; Kushwah et al., 2011; Kelly et al., 2012)</td>
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<td>Arabidopsis thaliana</td>
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<td>AtHXK1</td>
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<td>Type B M</td>
<td>Glc sensing PGD Photosynthetic gene repression (Jang et al., 1997; Kim et al., 2006; Karve et al., 2008)</td>
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<td>AtHXK3</td>
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<td>AT1G47840 15 and 16</td>
<td>Type A P</td>
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<td>AtHK1</td>
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<td>AT1G50460 17 and 18</td>
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<td>Growth Root hair development Mediates Glc-ethylene crosstalk Abiotic stress response (Claeyssen and Rivoal, 2007; Karve et al., 2008; Karve and Moore, 2009; Karve et al., 2012)</td>
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<td>Organism</td>
<td>Accession</td>
<td>Type</td>
<td>Function</td>
<td>Reference</td>
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<td>-------------------</td>
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<td>------------------------------------</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>AT4G37840</td>
<td>M</td>
<td>Abiotic stress response</td>
<td>(Claeyssen and Rivoal, 2007; Karve et al., 2008)</td>
</tr>
<tr>
<td><em>Solanum lycopersicum</em></td>
<td>AJ401153</td>
<td>M</td>
<td></td>
<td>(Damari-Weissler et al., 2006)</td>
</tr>
<tr>
<td>Tomato</td>
<td>AF208543</td>
<td>M</td>
<td></td>
<td>(Menu et al., 2001; Damari-Weissler et al., 2006)</td>
</tr>
<tr>
<td><em>Solanum chacoense</em></td>
<td>DQ056861</td>
<td>M</td>
<td></td>
<td>(Kandel-Kfir et al., 2006)</td>
</tr>
<tr>
<td><em>Solanum tuberosum</em></td>
<td>DQ056862</td>
<td>M</td>
<td></td>
<td>(Kandel-Kfir et al., 2006)</td>
</tr>
<tr>
<td><em>Sc HK2</em></td>
<td>DQ177440</td>
<td>ND</td>
<td></td>
<td>(Claeyssen et al., 2006)</td>
</tr>
<tr>
<td><em>St HK1</em></td>
<td>X94302</td>
<td>ND</td>
<td>Glc sensing</td>
<td>(Veramendi et al., 1999; Veramendi et al., 2002)</td>
</tr>
<tr>
<td><em>St HK2</em></td>
<td>AF106068</td>
<td>ND</td>
<td>Glc sensing</td>
<td>(Veramendi et al., 2002)</td>
</tr>
<tr>
<td><em>Nicotiana tabacum / benthamiana</em></td>
<td>AY553215</td>
<td>P</td>
<td>Plant growth</td>
<td>(Giese et al., 2005)</td>
</tr>
<tr>
<td><em>Nb HK1</em></td>
<td>AY286011</td>
<td>M</td>
<td>PCD Oxidative-stress resistance</td>
<td>(Kim et al., 2006; Sarowar et al., 2008)</td>
</tr>
<tr>
<td><em>Helianthus annuus</em></td>
<td>DQ835563</td>
<td>ND</td>
<td>Seed development</td>
<td>(Troncoso-Ponce et al., 2011)</td>
</tr>
<tr>
<td><em>Populus trichocarpa</em></td>
<td>XP_002325031</td>
<td>M</td>
<td>Glc sensing</td>
<td>(Karve et al., 2010)</td>
</tr>
<tr>
<td><em>Vitis vinifera L. cv. Cabernet Sauvignon</em></td>
<td>JN118544</td>
<td>ND</td>
<td></td>
<td>(Yu et al., 2012)</td>
</tr>
<tr>
<td><em>Spinacea oleracea</em></td>
<td>AF118132</td>
<td>M</td>
<td></td>
<td>(Wiese et al., 1999; Damari-Weissler et al., 2007)</td>
</tr>
<tr>
<td>Monocots</td>
<td></td>
<td></td>
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<tr>
<td><strong>OsHXK1</strong></td>
<td>DQ116383</td>
<td>C, N</td>
<td>(Cho et al., 2006a; Cheng et al., 2011)</td>
<td></td>
</tr>
<tr>
<td>(SEQ ID NOs: 47 and 48)</td>
<td></td>
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<tr>
<td><strong>OsHXK2</strong></td>
<td>DQ116384</td>
<td>Type B</td>
<td>(Cheng et al., 2011)</td>
<td></td>
</tr>
<tr>
<td>(SEQ ID NOs: 49 and 50)</td>
<td>M</td>
<td></td>
<td></td>
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<tr>
<td><strong>OsHXK3</strong></td>
<td>DQ116385</td>
<td>Type B</td>
<td>(Cheng et al., 2011)</td>
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<tr>
<td>(SEQ ID NOs: 51 and 52)</td>
<td>M</td>
<td></td>
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<tr>
<td><strong>OsHXK4</strong></td>
<td>DQ116386</td>
<td>Type A</td>
<td>(Cho et al., 2006a; Cheng et al., 2011)</td>
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<tr>
<td>(SEQ ID NOs: 53 and 54)</td>
<td>P</td>
<td></td>
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<td></td>
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<tr>
<td><strong>OsHXK5</strong></td>
<td>DQ116387</td>
<td>Type B Glc sensing</td>
<td>(Cho et al., 2009a; Cheng et al., 2011)</td>
<td></td>
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<tr>
<td>(SEQ ID NOs: 55 and 56)</td>
<td>M, N Photosynthetic gene repression</td>
<td></td>
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<tr>
<td>Rice</td>
<td></td>
<td>Shoot growth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Oryza sativa)</td>
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<td></td>
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<tr>
<td><strong>OsHXK6</strong></td>
<td>DQ116388</td>
<td>Type B Glc sensing</td>
<td>(Aki and Yanagisawa, 2009; Cho et al., 2009a; Cheng et al., 2011)</td>
<td></td>
</tr>
<tr>
<td>(SEQ ID NOs: 57 and 58)</td>
<td>M, N Photosynthetic gene repression</td>
<td></td>
<td></td>
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<td></td>
<td>Shoot growth</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>OsHXK7</strong></td>
<td>DQ116389</td>
<td>C, N</td>
<td>(Cho et al., 2006a; Cheng et al., 2011)</td>
<td></td>
</tr>
<tr>
<td>(SEQ ID NOs: 59 and 60)</td>
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<td></td>
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<td>DQ116390</td>
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<tr>
<td><strong>OsHXK8</strong></td>
<td>(SEQ ID NOs: 61 and 62)</td>
<td>C, N</td>
<td>(Cheng et al., 2011)</td>
<td></td>
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<td>DQ116391</td>
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<tr>
<td><strong>OsHXK9</strong></td>
<td>(SEQ ID NOs: 63 and 64)</td>
<td>Type B</td>
<td>(Cheng et al., 2011)</td>
<td></td>
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<tr>
<td>DQ116392</td>
<td>M</td>
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<td></td>
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<tr>
<td><strong>OsHXK10</strong></td>
<td>(SEQ ID NOs: 65 and 66)</td>
<td>Type B</td>
<td>(Xu et al., 2008; Cheng et al., 2011)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M Pollen germination</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>and/or C</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

| Sorghum (Sorghum bicolor) | "SbHXK3" | XM_0024592072 | Type B | No Glc sensing role | (Karve et al., 2010) |
| (SEQ ID NOs: 67 and 68) | M | | | | |
| **SbHXK8** | XM_002454982 | C | (Karve et al., 2010) |
| (SEQ ID NOs: 69 and 70) | | | | | |

| Wheat (Triticum aestivum) | "HXK" | AY974231 | ND | Controls triose phosphate/phosphate translocation | (Sun et al., 2006) |
| (SEQ ID NOs: 71 and 72) | | | | | |
### Lycophytes

<table>
<thead>
<tr>
<th>Species</th>
<th>Accession No.</th>
<th>Isoform</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spike moss (Selaginella mollendorffii)</td>
<td>SmHXK3 26000047</td>
<td>C</td>
<td>Glc sensing</td>
<td>(Karve et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>SmHXK5 57.357.1</td>
<td>C</td>
<td></td>
<td>(Karve et al., 2010)</td>
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</table>

### Bryophytes

<table>
<thead>
<tr>
<th>Species</th>
<th>Accession No.</th>
<th>Isoform</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moss (Physcomitrella patens)</td>
<td>PpHXK1 AY260967 (SEQ ID NOs: 73 and 74)</td>
<td>Type A</td>
<td>Filamentous type and growth</td>
<td>(Olsson et al., 2003; Thelander et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>PpHXK2 XM_00 1784578 (SEQ ID NOs: 75 and 76)</td>
<td>Type B</td>
<td>M, P</td>
<td>(Nilsson et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>PpHXK3 XM_00 1784282 (SEQ ID NOs: 77 and 78)</td>
<td>Type B</td>
<td>M, P</td>
<td>(Nilsson et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>PpHXK4 XM_00 1760896 (SEQ ID NOs: 79 and 80)</td>
<td>Type C</td>
<td>C, N</td>
<td>(Nilsson et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>PpHXK5 XM_00 1762899 (SEQ ID NOs: 81 and 82)</td>
<td>Type A</td>
<td>P</td>
<td>(Nilsson et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>PpHXK6 XM_00 1754096 (SEQ ID NOs: 83 and 84)</td>
<td>Type A</td>
<td>P</td>
<td>(Nilsson et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>PpHXK7 XM_00 1752177 (SEQ ID NOs: 85 and 86)</td>
<td>Type B</td>
<td>M, P</td>
<td>(Nilsson et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>PpHXK8 XM_00 1770125 (SEQ ID NOs: 87 and 88)</td>
<td>Type B</td>
<td>M, P</td>
<td>(Nilsson et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>PpHXK9 XM_00 1776713 (SEQ ID NOs: 89 and 90)</td>
<td>Type D</td>
<td>M</td>
<td>(Nilsson et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>PpHXKW XM_00 1779426 (SEQ ID NOs: 91 and 92)</td>
<td>Type D</td>
<td>M</td>
<td>(Nilsson et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>PpHXK11 XM_00 1779426 (SEQ ID NOs: 93 and 94)</td>
<td>Type D</td>
<td>M, P</td>
<td>(Nilsson et al., 2011)</td>
</tr>
</tbody>
</table>


*Joint Genome Institute- Selaginella moellendorffii v1.0.*
As mentioned, the HXK sequence may be naturally occurring or artificially generated (e.g., codon-optimized) according to the intended use.

According to a specific embodiment, modulating the activity or expression of HXK refers to upregulating the activity or expression which results in reduction of stomatal conductance. Upregulating can be by at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or more, say 90% or even 100%, as compared to hexokinase expression or activity in a similar cell of the same plant species, growth conditions and developmental stage (e.g., wild-type (WT) plant).

As mentioned, upregulation of hexokinase activity or expression in a guard-cell specific manner has a number of advantages in crop plants and vegetables farming.

Thus, the present inventors have shown that upregulation of HXK in a guard-cell specific manner decreases stomata aperture and conductance (without affecting photosynthesis), improves plant's water use efficiency, thereby increasing plant's tolerance to drought, and overall increases plants vigor, biomass or yield (under stress or optimal growth conditions). Likewise, plants expressing HXK in a guard-cell specific manner are tolerant to salinity stress. It is appreciated that water are taken up (soaked) by plants as a result of the difference between water potential in the air and within the plants. This difference is termed vapor pressure deficit (VPD). The driving force of soaking water from the ground is the VPD. Higher VPD - the greater is the force. Yet, when the stomata are partially closed, the effect of VPD is lowered and less water is being taken up by the plant. In that case, the plant will take less salt from the ground and will be less affected. The present teachings have also an unprecedented impact on the tolerance of plants to biotic stress. Many human and plant pathogens such as bacteria and fungi, invade plants via the stomata (see for Example Kroupitski et al. Applied and Environmental Microbiology 2009 6076-6086 teaching that Salmonella enteric internalizes in leaves via open stomata). Not only does the stomata allow easy entrance, but also serve as good environment for attracting the pathogens by the accumulation of sugars near the guard cells when the stomata is open. Indeed, the present inventors have observed reduced fungi and bacteria infections in plants with high expression of HXK (not shown).

Alternatively or additionally, the present teachings can also be employed towards imparting the plant with a tolerance to temperature stress (heat or cold). For instance,
plants expressing high levels of HXK in a guard cell specific manner are expected to exhibit extended heat and cold resistance with regard to fruit setting. Pollen development and germination are sensitive to heat and cold, most likely due to perturbation of sugar metabolism. It is suggested that during heat stress less sugars are being earned toward the pollen grains (and other sink tissues as well) since most of the water is transpired through the stomata. According to the present teachings, when less water is transpired through the stomata so then more water is available for sugar transport in the phloem. That may impart resistance to temperature stress (e.g., heat) thereby allowing production of viable pollen grains.

Alternatively or additionally, the present teachings can be employed towards prevention of blossom end rot (BER). BER is a visible physiological damage that affects many crops such as tomato, eggplants, pepper, melon and many more. BER happens mainly under heat and water stress. It is now suggested that under such conditions, most of the water is transpired and less water is available to carry sugars, minerals and ions toward the fruits. Accordingly, lowering transpiration may allocate more water carrying more sugars, minerals and ions toward the fruits and other sink tissues (Nikinma et al. 2012 Plant, Cell and Environment 2012 1-15). BER is determined by the percentage of fruits that exhibit visible or detectable rot (physical damage) on the fruit. BER prevention means lowering the percentage of fruits with BER.

Thus, according to an exemplary embodiment the present teachings can be used to increase biomass, vigor or yield of a plant.

As used herein the phrase "plant yield" refers to the amount (e.g., as determined by weight or size) or quantity (numbers) of tissues or organs produced per plant or per growing season. Hence increased yield could affect the economic benefit one can obtain from the plant in a certain growing area and/or growing time.

It should be noted that a plant yield can be affected by various parameters including, but not limited to, plant biomass; plant vigor; growth rate; seed yield; seed or grain quantity; seed or grain quality; oil yield; content of oil, starch and/or protein in harvested organs (e.g., seeds, fruits or vegetative parts of the plant); number of flowers (florets) per panicle (expressed as a ratio of number of filled seeds over number of primary panicles); harvest index; number of plants grown per area; number and size of
harvested organs per plant and per area; number of plants per growing area (density); number of harvested organs in field; total leaf area; carbon assimilation and carbon partitioning (the distribution/allocation of carbon within the plant); resistance to shade; number of harvestable organs (e.g. seeds), seeds per pod, weight per seed; and modified architecture [such as increase stalk diameter, thickness or improvement of physical properties (e.g. elasticity)] .

As used herein the phrase "seed yield" refers to the number or weight of the seeds per plant, seeds per pod, or per growing area or to the weight of a single seed, or to the oil extracted per seed. Hence seed yield can be affected by seed dimensions (e.g., length, width, perimeter, area and/or volume), number of (filled) seeds and seed filling rate and by seed oil content. Hence increase seed yield per plant could affect the economic benefit one can obtain from the plant in a certain growing area and/or growing time; and increase seed yield per growing area could be achieved by increasing seed yield per plant, and/or by increasing number of plants grown on the same given area.

The term "seed" (at times referred to as "grain" or "kernel") as used herein refers to a small embryonic plant enclosed in a covering called the seed coat (usually with some stored food), the product of the ripened ovule of gymnosperm and angiosperm plants which occurs after fertilization and some growth within the mother plant. The seed may be a hybrid seed or a homozygous line.

The phrase "oil content" as used herein refers to the amount of lipids in a given plant organ, either the seeds (seed oil content) or the vegetative portion of the plant (vegetative oil content) and is typically expressed as percentage of dry weight (10 % humidity of seeds) or wet weight (for vegetative portion).

It should be noted that oil content is affected by intrinsic oil production of a tissue (e.g., seed, fruit, vegetative portion), as well as the mass or size of the oil-producing tissue per plant or per growth period.

In one embodiment, increase in oil content of the plant can be achieved by increasing the size/mass of a plant's tissue(s) which comprise oil per growth period. Thus, increased oil content of a plant can be achieved by increasing the yield, growth rate, biomass and vigor of the plant.
As used herein the phrase "plant biomass" refers to the amount (e.g., measured in grams of air-dry tissue) of a tissue produced from the plant in a growing season, which could also determine or affect the plant yield or the yield per growing area. An increase in plant biomass can be in the whole plant or in parts thereof such as aboveground (harvestable) parts, fruit biomass, vegetative biomass, roots and seeds.

As used herein the phrase "growth rate" refers to the increase in plant organ/tissue size per time (can be measured in cm² per day).

As used herein the phrase "plant vigor" refers to the amount (measured by weight) of tissue produced by the plant in a given time. Hence increased vigor could determine or affect the plant yield or the yield per growing time or growing area. In addition, early vigor (seed and/or seedling) results in improved field stand.

It should be noted that a plant yield can be determined under stress (e.g., abiotic stress) and/or non-stress (normal) conditions. It is contemplated herein that yield, vigor or biomass of the plant expressing the HXK in a guard cell-specific manner is increased as compared to that of wild-type plant (not expressing said HXK) under stress or non-stressed conditions.

As used herein, the phrase "non-stress conditions" (or normal or optimal as referred to herein) refers to the growth conditions (e.g., water, temperature, light-dark cycles, humidity, salt concentration, fertilizer concentration in soil, nutrient supply such as nitrogen, phosphorous and/or potassium), that do not significantly go beyond the everyday climatic and other abiotic conditions that plants may encounter, and which allow optimal growth, metabolism, reproduction and/or viability of a plant at any stage in its life cycle (e.g., in a crop plant from seed to a mature plant and back to seed again). Persons skilled in the art are aware of normal soil conditions and climatic conditions for a given plant in a given geographic location. It should be noted that while the non-stress conditions may include some mild variations from the optimal conditions (which vary from one type/species of a plant to another), such variations do not cause the plant to cease growing without the capacity to resume growth.

As mentioned increased yield can be under non-stress conditions or abiotic/biotic stress conditions.

The phrase "abiotic stress" as used herein refers to any adverse effect on metabolism, growth, reproduction and/or viability of a plant. Accordingly, abiotic
stress can be induced by suboptimal environmental growth conditions such as, for example, salinity, water deprivation, flooding, freezing, low or high temperature (i.e., cold or heat), heavy metal toxicity, anaerobiosis, nutrient deficiency, atmospheric pollution or UV irradiation.

The phrase "abiotic stress tolerance" as used herein refers to the ability of a plant to endure an abiotic stress without suffering a substantial alteration in metabolism, growth, productivity and/or viability.

As used herein the phrase "water use efficiency (WUE)" refers to the level of organic matter produced per unit of water consumed by the plant, i.e., the dry weight of a plant in relation to the plant's water use, e.g., the biomass produced per unit transpiration.

Similarly "biotic stress" refers to stress that occurs as a result of damage done to plants by other living organisms, such as bacteria, viruses, fungi, parasites.

Upregulation of HXK in a guard-cell specific manner can be used to remedy any of the aforementioned conditions and to improve plants performance overall. Thus, upregulation of the HXK can be effected by expressing an exogenous polynucleotide encoding HXK in the plant in a guard-cell specific manner.

The phrase "expressing within the plant an exogenous polynucleotide encoding HXK" as used herein refers to upregulating the expression level of an exogenous polynucleotide encoding an HXK polypeptide within the plant by introducing the exogenous polynucleotide into a plant cell or plant and expressing by recombinant means, as further described herein below.

As used herein "expressing" refers to expression at the mRNA and polypeptide level. It will be appreciated that for silencing the expression is at the mRNA level alone (silencing mechanisms of HXK are described further hereinbelow).

As used herein, the phrase "exogenous polynucleotide" refers to a heterologous nucleic acid sequence which may not be naturally expressed within the plant or which overexpression in the plant is desired. The exogenous polynucleotide may be introduced into the plant in a stable or transient manner, so as to produce a ribonucleic acid (RNA) molecule and/or a polypeptide molecule. It should be noted that the exogenous polynucleotide may comprise a nucleic acid sequence which is identical or partially homologous to an endogenous nucleic acid sequence of the plant.
The term "endogenous" as used herein refers to any polynucleotide or polypeptide which is present and/or naturally expressed within a plant or a cell thereof.

According to the invention, the exogenous polynucleotide of the invention comprises a nucleic acid sequence encoding a polypeptide having an amino acid sequence of a hexokinase.

According to a specific embodiment the amino acid sequence of the HXK polypeptide (encoded from the exogenous polynucleotide) is at least about, 30 %, 40 % or 50%, or at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80 %, at least about 81 %, at least about 82 %, at least about 83 %, at least about 84 %, at least about 85 %, at least about 86 %, at least about 87 %, at least about 88 %, at least about 89 %, at least about 90 %, at least about 91 %, at least about 92 %, at least about 93 %, at least about 94 %, at least about 95 %, at least about 96 %, at least about 97 %, at least about 98 %, at least about 99 %, or more say 100 % homologous to the amino acid sequence selected from the group consisting of SEQ ID NOs: 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92 and 94, as long as its hexokinase activity is maintained as described above.

Homology (e.g., percent homology, identity + similarity) can be determined using any homology comparison software, including for example, the BlastP or TBLASTN software of the National Center of Biotechnology Information (NCBI) such as by using default parameters, when starting from a polypeptide sequence; or the tBLASTX algorithm (available via the NCBI) such as by using default parameters, which compares the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database.

According to some embodiments of the invention, the term "homology" or "homologous" refers to identity of two or more nucleic acid sequences; or identity of two or more amino acid sequences.

Homologous sequences include both orthologous and paralogous sequences. The term "paralogous" relates to gene-duplications within the genome of a species leading to paralogous genes. The term "orthologous" relates to homologous genes in different organisms due to ancestral relationship.
One option to identify orthologues in monocot plant species is by performing a reciprocal blast search. This may be done by a first blast involving blasting the sequence-of-interest against any sequence database, such as the publicly available NCBI database which may be found at: Hypertext Transfer Protocol://World Wide Web (dot) ncbi (dot) nlm (dot) nih (dot) gov. If orthologues in rice were sought, the sequence-of-interest would be blasted against, for example, the 28,469 full-length cDNA clones from Oryza sativa Nipponbare available at NCBI. The blast results may be filtered. The full-length sequences of either the filtered results or the non-filtered results are then blasted back (second blast) against the sequences of the organism from which the sequence-of-interest is derived. The results of the first and second blasts are then compared. An orthologue is identified when the sequence resulting in the highest score (best hit) in the first blast identifies in the second blast the query sequence (the original sequence-of-interest) as the best hit. Using the same rational a paralogue (homolog to a gene in the same organism) is found. In case of large sequence families, the ClustalW program may be used [Hypertext Transfer Protocol://World Wide Web (dot) ebi (dot) ac (dot) uk/Tools/clustalw2/index (dot) html], followed by a neighbor-joining tree (Hypertext Transfer Protocol://en (dot) wikipedia (dot) org/wiki/Neighbor-joining) which helps visualizing the clustering.

According to some embodiments of the invention, the exogenous polynucleotide of the invention encodes a polypeptide having an amino acid sequence at least about 30 %, 40 %, 50 %, 60 %, 70 % or at least about 80 %, at least about 81 %, at least about 82 %, at least about 83 %, at least about 84 %, at least about 85 %, at least about 86 %, at least about 87 %, at least about 88 %, at least about 89 %, at least about 90 %, at least about 91 %, at least about 92 %, at least about 93 %, at least about 94 %, at least about 95 %, at least about 96 %, at least about 97 %, at least about 98 %, at least about 99 %, or more say 100 % identical to the amino acid sequence selected from the group consisting of 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92 and 94 as long as the hexokinase activity of the protein (as described above) is maintained.

As used herein the term "polynucleotide" refers to a single or double stranded nucleic acid sequence which is isolated and provided in the form of an RNA sequence, a
complementary polynucleotide sequence (cDNA), a genomic polynucleotide sequence and/or a composite polynucleotide sequences (e.g., a combination of the above).

The term "isolated" refers to at least partially separated from the natural environment e.g., from a plant cell.

As used herein the phrase "complementary polynucleotide sequence" refers to a sequence, which results from reverse transcription of messenger RNA using a reverse transcriptase or any other RNA dependent DNA polymerase. Such a sequence can be subsequently amplified in vivo or in vitro using a DNA dependent DNA polymerase.

As used herein the phrase "genomic polynucleotide sequence" refers to a sequence derived (isolated) from a chromosome and thus it represents a contiguous portion of a chromosome.

As used herein the phrase "composite polynucleotide sequence" refers to a sequence, which is at least partially complementary and at least partially genomic. A composite sequence can include some exonal sequences required to encode the polypeptide of the present invention, as well as some intronic sequences inteiposing therebetween. The intronic sequences can be of any source, including of other genes, and typically will include conserved splicing signal sequences. Such intronic sequences may further include cis acting expression regulatory elements.

Nucleic acid sequences encoding the HXK polypeptides of the present invention may be optimized for expression. Examples of such sequence modifications include, but are not limited to, an altered G/C content to more closely approach that typically found in the plant species of interest, and the removal of codons atypically found in the plant species commonly referred to as codon optimization.

The phrase "codon optimization" refers to the selection of appropriate DNA nucleotides for use within a structural gene or fragment thereof that approaches codon usage within the plant of interest. Therefore, an optimized gene or nucleic acid sequence refers to a gene in which the nucleotide sequence of a native or naturally occurring gene has been modified in order to utilize statistically-preferred or statistically-favored codons within the plant. The nucleotide sequence typically is examined at the DNA level and the coding region optimized for expression in the plant species determined using any suitable procedure, for example as described in Sardana et al. (1996, Plant Cell Reports 15:677-681). In this method, the standard deviation of
codon usage, a measure of codon usage bias, may be calculated by first finding the
squared proportional deviation of usage of each codon of the native gene relative to that
of highly expressed plant genes, followed by a calculation of the average squared
deviation. The formula used is: \[ \text{SDCU} = \frac{1}{n} \sum \left( \frac{x_i - y_i}{N} \right)^2 \]
where \( x_i \) refers to the frequency of usage of codon \( i \) in the gene of interest and \( y_i \) refers to the frequency of usage of codon \( i \) in highly expressed plant genes. A Table of codon usage from highly
expressed genes of dicotyledonous plants is compiled using the data of Murray et al.

One method of optimizing the nucleic acid sequence in accordance with the
prefixed codon usage for a particular plant cell type is based on the direct use, without
performing any extra statistical calculations, of codon optimization Tables such as those
provided on-line at the Codon Usage Database through the NIAS (National Institute of
Agrobiological Sciences) DNA bank in Japan (Hypertext Transfer Protocol://World
Wide Web (dot) kazusa (dot) or (dot) jp/codon/). The Codon Usage Database contains
codon usage tables for a number of different species, with each codon usage Table
having been statistically determined based on the data present in Genbank.

By using the above Tables to determine the most prefixed or most favored
codons for each amino acid in a particular species (for example, rice), a naturally-
occuring nucleotide sequence encoding a protein of interest can be codon optimized for
that particular plant species. This is effected by replacing codons that may have a low
statistical incidence in the particular species genome with coresponding codons, in
regard to an amino acid, that are statistically more favored. However, one or more less-
favored codons may be selected to delete existing restriction sites, to create new ones at
potentially useful junctions (5’ and 3’ ends to add signal peptide or termination cassettes,
internal sites that might be used to cut and splice segments together to produce a correct
full-length sequence), or to eliminate nucleotide sequences that may negatively effect
liRNA stability or expression.

The naturally-occuring encoding nucleotide sequence may already, in advance
of any modification, contain a number of codons that correspond to a statistically-
favored codon in a particular plant species. Therefore, codon optimization of the native
nucleotide sequence may comprise determining which codons, within the native
nucleotide sequence, are not statistically-favored with regards to a particular plant, and modifying these codons in accordance with a codon usage table of the particular plant to produce a codon optimized derivative. A modified nucleotide sequence may be fully or partially optimized for plant codon usage provided that the protein encoded by the modified nucleotide sequence is produced at a level higher than the protein encoded by the corresponding naturally occurring or native gene. Construction of synthetic genes by altering the codon usage is described in for example PCT Patent Application 93/07278.

The term "plant" as used herein encompasses whole plants, ancestors and progeny of the plants and plant parts (those which comprise stomata but not necessarily), including seeds, shoots, stems, roots (including tubers), and plant cells, tissues and organs. The plant may be in any form including suspension cultures, embryos, meristematic regions, callus tissue, leaves, gametophytes, sporophytes, pollen, and microspores.

According to some embodiments of the invention the plant is a dicotyledonous plant.

According to some embodiments of the invention the plant is a monocotyledonous plant.

Plants that are particularly useful in the methods of the invention include all plants which belong to the superfamily Viridiplantae, in particular monocotyledonous and dicotyledonous plants including a fodder or forage legume, ornamental plant, food crop, tree, or shrub selected from the list comprising Acacia spp., Acer spp., Actinidia spp., Aesculus spp., Agathis australis, Albizia amara, Alsophila tricolor, Andropogon spp., Arachis spp., Areca catechu, Astelia fragrans, Astragalus cicer, Baikiaea plurijuga, Betula spp., Brassica spp., Bruguiera gymnorrhiza, Burkea africana, Butea frondosa, Cadaba farinosa, Calliandra spp., Camellia sinensis, Canna indica, Capsicum spp., Cassia spp., Centroema pubescens, Chacoomeles spp., Cinnamomum cassia, Coffea arabica, Colophospermum mopane, Coronillia varia, Cotoneaster serotina, Crataegus spp., Cucumis spp., Cupressus spp., Cyathea dealbata, Cydonia oblonga, Cryptomeria japonica, Cymbopogon spp., Cynthea dealbata, Cydonia oblonga, Dalbergia monetaria, Davallia divaricata, Desmodium spp., Dicksonia squarosa, Dibeteropogon amplectens, Dioclea spp, Dolichos spp., Dorycnium rectum, Echinochloa pyramidalis, Ehraffia spp.,

According to some embodiments of the invention, the plant used by the method of the invention is a crop plant such as rice, maize, wheat, barley, peanut, potato, sesame, olive tree, palm oil, banana, soybean, sunflower, canola, sugarcane, alfalfa, millet, leguminosae (bean, pea), flax, lupinus, rapeseed, tobacco, poplar and cotton.
According to some embodiments of the invention, the plant is a tomato or a banana.

According to some embodiments of the invention, expressing the exogenous polynucleotide of the invention within the plant is effected by introducing into a cell of the plant (e.g., transforming one or more cells of the plant) an exogenous polynucleotide encoding the HXK under a cis-acting regulatory element for driving expression of the HXK in a guard-cell specific manner, followed by generating a mature plant from the transformed cells and cultivating the mature plant under conditions suitable for expressing the exogenous polynucleotide within the mature plant.

Thus, there is provided a plant expression construct comprising a nucleic acid sequence encoding a hexokinase under a transcriptional control of a guard cell-specific cis-acting regulatory element and methods which make use of same.

There is also provided a method of decreasing plant stomata conductance, the method comprising introducing into a cell of a plant the above-described nucleic acid construct, thereby decreasing the stomata conductance of the plant.

Alternatively or additionally there is provided a method of increasing water use efficiency of a plant, the method comprising introducing into a cell of the plant the above-described nucleic acid construct, thereby increasing water use efficiency of the plant.

Alternatively or additionally there is provided a method of increasing tolerance of a plant to drought, salinity or temperature stress, the method comprising introducing into a cell of the plant the above-described nucleic acid construct, thereby increasing tolerance of the plant to drought, salinity or temperature stress.

Alternatively or additionally there is provided a method of increasing biotic stress tolerance of a plant, the method comprising introducing into a cell of the plant the above-described nucleic acid construct, thereby increasing biotic stress tolerance of the plant.

Alternatively or additionally there is provided a method of increasing biomass, vigor or yield of a plant, the method comprising introducing into a cell of the plant the nucleic acid construct, thereby increasing the biomass, vigor or yield of the plant.

According to some embodiments of the invention, the transformation is effected by introducing to the plant cell a nucleic acid construct which includes the exogenous
polynucleotide of some embodiments of the invention encoding the HXK (as described above) and a guard cell-specific cis-acting regulatory element. Further details of suitable transformation approaches are provided hereinbelow.

As used herein "guard-cell specific cis-acting regulatory element" refers to the ability of a transcriptional element to drive expression of the nucleic acid sequence under its regulation (e.g., HXK) only in guard cells, leaving the rest of the tissues in the plant unmodified by transgene expression (e.g., more than 90% of the mRNA is expressed in the tissue of interest, as detected by RT-PCR). Tissue-specific cis-acting regulatory elements may be induced by endogenous or exogenous factors, so they can be classified as inducible promoters as well. In other cases they are constitutively expressed.

A coding nucleic acid sequence (e.g., HXK) is "operably linked" to a regulatory sequence (e.g., guard-cell specific promoter) if the regulatory sequence is capable of exerting a regulatory effect on the coding sequence linked thereto.

According to some embodiments of the invention the cis-acting regulatory element is a promoter.

As used herein, the term "promoter" refers to a region of DNA which lies upstream of the transcriptional initiation site of a gene to which RNA polymerase binds to initiate transcription of RNA. The promoter controls where (e.g., which portion of a plant) and/or when (e.g., at which stage or condition in the lifetime of an organism) the gene is expressed.

Examples of guard-cell specific promoters include, but are not limited to the promoters listed in Table 2 below and the KST1 promoter used in the Examples section (SEQ ID NO: 108).

<p>| Table 2 |
|---|---|---|---|---|---|
| <strong>Promoter</strong> | <strong>Species</strong> | <strong>Accession n.</strong> | <strong>Verification method</strong> | <strong>Ref.</strong> | <strong>Comments</strong> |
| AtMYB61 promoter | Arabidopsis thaliana | AT1G09540 (SEQ ID NO: 95) | GFP | (Liang et al., 2005) | Specific expression in GC |</p>
<table>
<thead>
<tr>
<th></th>
<th>Promoter</th>
<th>Species</th>
<th>Accession No.</th>
<th>Reporter</th>
<th>Expression Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Atlg22690-promoter (pGCl)</td>
<td>Arabidopsis thaliana</td>
<td>Atlg22690 (SEQ ID NO: 96)</td>
<td>GFP based on FRET with calcium reporter / GUS</td>
<td>Specific expression in GC</td>
</tr>
<tr>
<td>3</td>
<td>AtMYB60 promoter</td>
<td>Arabidopsis thaliana</td>
<td>Atlg088 10 (SEQ ID NO: 97)</td>
<td>GUS, GFP</td>
<td>Specific expression in GC</td>
</tr>
<tr>
<td>4</td>
<td>R2R3 MYB60 transcription factor promoter</td>
<td>Vitis vinifera L.</td>
<td>ACF2 1938 (SEQ ID NO: 98)</td>
<td>GUS</td>
<td>Specific expression in GC</td>
</tr>
<tr>
<td>5</td>
<td>H1C (High carbon dioxide) promoter</td>
<td>Arabidopsis thaliana</td>
<td>AT2G46720 (SEQ ID NO: 99)</td>
<td>GUS</td>
<td>Specific expression in GC</td>
</tr>
<tr>
<td>6</td>
<td>CYTOCHROME E P450 86A2 (CYP634A2) monooxygenase promoter (pCYP)</td>
<td>Arabidopsis thaliana</td>
<td>At4g00360 (SEQ ID NO: 100)</td>
<td>GFP</td>
<td>Specific expression in GC</td>
</tr>
<tr>
<td>7</td>
<td>ADP-glucose pyrophosphorylase (AGPase) Promoter</td>
<td>Solanum tuberosum</td>
<td>X75017 (Promoter seq.) (SEQ ID NO: 101)</td>
<td>GUS</td>
<td>Specific expression in GC. Given that it is 0.3 Kb 5' proximal promoter - exclusive GC expression.</td>
</tr>
<tr>
<td>8</td>
<td>KAT1 promoter</td>
<td>Arabidopsis thaliana</td>
<td>AT5G46240 (gene), U25088 (promoter+gene seq.) (SEQ ID NO: 102)</td>
<td>GUS</td>
<td>Specific expression in GC. However, was detected also in vascular tissue of roots</td>
</tr>
<tr>
<td>9</td>
<td>Myrosinase-Thioglucoside glucohydrolase 1 (TGG1) promoter</td>
<td>Arabidopsis thaliana</td>
<td>At5g26000 (SEQ ID NO: 103)</td>
<td>GUS, GFP</td>
<td>Specific expression in GC. Distinct expression in phloem</td>
</tr>
<tr>
<td>10</td>
<td>rhal promoter</td>
<td>Arabidopsis thaliana</td>
<td>AT5G45 130 (SEQ ID NO: 104)</td>
<td>GUS</td>
<td>Mainly expressed (non-specific) in GC</td>
</tr>
</tbody>
</table>
The nucleic acid constiaict of some embodiments of the invention can further include an appropriate selectable marker and/or an origin of replication. According to some embodiments of the invention, the nucleic acid constiaict utilized is a shuttle vector, which can propagate both in E. coli (wherein the constiaict comprises an appropriate selectable marker and origin of replication) and be compatible with propagation in cells. The constiaict according to the present invention can be, for example, a plasmid, a bacmid, a phagemid, a cosmid, a phage, a vims or an artificial chromosome.

The nucleic acid constiaict of some embodiments of the invention can be utilized to stably or transiently transform plant cells. In stable transformation, the exogenous polynucleotide is integrated into the plant genome and as such it represents a stable and inherited trait. In transient transformation, the exogenous polynucleotide is expressed by the cell transformed but it is not integrated into the genome and as such it represents a transient trait.


The Agrobacterium system includes the use of plasmid vectors that contain defined DNA segments that integrate into the plant genomic DNA. Methods of inoculation of the plant tissue vary depending upon the plant species and the Agrobacterium delivery system. A widely used approach is the leaf disc procedure which can be performed with any tissue explant that provides a good source for initiation of whole plant differentiation. See, e.g., Horsch et al. in Plant Molecular Biology Manual A5, Kluwer Academic Publishers, Dordrecht (1988) p. 1-9. A supplementary approach employs the Agrobacterium delivery system in combination with vacuum infiltration. The Agrobacterium system is especially viable in the creation of transgenic dicotyledonous plants.

There are various methods of direct DNA transfer into plant cells. In electroporation, the protoplasts are briefly exposed to a strong electric field. In microinjection, the DNA is mechanically injected directly into the cells using very small micropipettes. In microparticle bombardment, the DNA is adsorbed on microparticles such as magnesium sulfate crystals or tungsten particles, and the microparticles are physically accelerated into cells or plant tissues.

Following stable transformation plant propagation is exercised. The most common method of plant propagation is by seed. Regeneration by seed propagation, however, has the deficiency that due to heterozygosity there is a lack of uniformity in
the crop, since seeds are produced by plants according to the genetic variances governed
by Mendelian rules. Basically, each seed is genetically different and each will grow
with its own specific traits. Therefore, it is preferred that the transformed plant be
produced such that the regenerated plant has the identical traits and characteristics of the
parent transgenic plant. Therefore, it is preferred that the transformed plant be
regenerated by micropropagation which provides a rapid, consistent reproduction of the
transformed plants.

Micropropagation is a process of growing new generation plants from a single
piece of tissue that has been excised from a selected parent plant or cultivar. This
process permits the mass reproduction of plants having the preferred tissue expressing
the fusion protein. The new generation plants which are produced are genetically
identical to, and have all of the characteristics of, the original plant. Micropropagation
allows mass production of quality plant material in a short period of time and offers a
rapid multiplication of selected cultivars in the preservation of the characteristics of the
original transgenic or transformed plant. The advantages of cloning plants are the speed
of plant multiplication and the quality and uniformity of plants produced.

Micropropagation is a multi-stage procedure that requires alteration of culture
medium or growth conditions between stages. Thus, the micropropagation process
involves four basic stages: Stage one, initial tissue culturing; stage two, tissue culture
multiplication; stage three, differentiation and plant formation; and stage four,
greenhouse culturing and hardening. During stage one, initial tissue culturing, the tissue
culture is established and certified contaminant-free. During stage two, the initial tissue
culture is multiplied until a sufficient number of tissue samples are produced to meet
production goals. During stage three, the tissue samples grown in stage two are divided
and grown into individual plantlets. At stage four, the transformed plantlets are
transferred to a greenhouse for hardening where the plants' tolerance to light is
gradually increased so that it can be grown in the natural environment.

According to some embodiments of the invention, the transgenic plants are
generated by transient transformation of leaf cells, meristematic cells or the whole plant.

Transient transformation can be effected by any of the direct DNA transfer
methods described above or by viral infection using modified plant viruses.
Viruses that have been shown to be useful for the transformation of plant hosts include CaMV, Tobacco mosaic virus (TMV), brome mosaic vims (BMV) and Bean Common Mosaic Vims (BV or BCMV). Transformation of plants using plant viruses is described in U.S. Pat. No. 4,855,237 (bean golden mosaic vims; BGV), EP-A 67,553 (TMV), Japanese Published Application No. 63-14693 (TMV), EPA 194,809 (BV), EPA 278,667 (BV); and Gluzman, Y. et al., Communications in Molecular Biology: Viral Vectors, Cold Spring Harbor Laboratory, New York, pp. 172-189 (1988). Pseudovims particles for use in expressing foreign DNA in many hosts, including plants are described in WO 87/06261.

According to some embodiments of the invention, the vims used for transient transformations is avimlent and thus is incapable of causing severe symptoms such as reduced growth rate, mosaic, ring spots, leaf roll, yellowing, streaking, pox formation, tumor formation and pitting. A suitable avimlent vims may be a naturally occurring avimlent vims or an artificially attenuated vims. Vims attenuation may be effected by using methods well known in the art including, but not limited to, sub-lethal heating, chemical treatment or by directed mutagenesis techniques such as described, for example, by Kurihara and Watanabe (Molecular Plant Pathology 4:259-269, 2003), Galon et al. (1992), Atreya et al. (1992) and Huet et al. (1994).

Suitable vims strains can be obtained from available sources such as, for example, the American Type culture Collection (ATCC) or by isolation from infected plants. Isolation of viruses from infected plant tissues can be effected by techniques well known in the art such as described, for example by Foster and Tatlor, Eds. "Plant Virology Protocols: From Vims Isolation to Transgenic Resistance (Methods in Molecular Biology (Humana Pr), Vol 81)", Humana Press, 1998. Briefly, tissues of an infected plant believed to contain a high concentration of a suitable vims, preferably young leaves and flower petals, are ground in a buffer solution (e.g., phosphate buffer solution) to produce a vims infected sap which can be used in subsequent inoculations.

When the vims is a DNA vims, suitable modifications can be made to the vims itself. Alternatively, the vims can first be cloned into a bacterial plasmid for ease of constructing the desired viral vector with the foreign DNA. The vims can then be excised from the plasmid. If the vims is a DNA vims, a bacterial origin of replication can be attached to the viral DNA, which is then replicated by the bacteria. Transcription and translation of this DNA will produce the coat protein which will encapsidate the viral DNA. If the vims is an RNA vims, the vims is generally cloned as a cDNA and inserted into a plasmid. The plasmid is then used to make all of the constructions. The RNA vims is then produced by transcribing the viral sequence of the plasmid and translation of the viral genes to produce the coat protein(s) which encapsidate the viral RNA.

In one embodiment, a plant viral polynucleotide is provided in which the native coat protein coding sequence has been deleted from a viral polynucleotide, a non-native plant viral coat protein coding sequence and a non-native promoter, preferably the subgenomic promoter of the non-native coat protein coding sequence, capable of expression in the plant host, packaging of the recombinant plant viral polynucleotide, and ensuring a systemic infection of the host by the recombinant plant viral polynucleotide, has been inserted. Alternatively, the coat protein gene may be inactivated by insertion of the non-native polynucleotide sequence within it, such that a protein is produced. The recombinant plant viral polynucleotide may contain one or more additional non-native subgenomic promoters. Each non-native subgenomic promoter is capable of transcribing or expressing adjacent genes or polynucleotide sequences in the plant host and incapable of recombination with each other and with native subgenomic promoters. Non-native (foreign) polynucleotide sequences may be inserted adjacent the native plant viral subgenomic promoter or the native and a non-native plant viral subgenomic promoters if more than one polynucleotide sequence is included. The non-native polynucleotide sequences are transcribed or expressed in the host plant under control of the subgenomic promoter to produce the desired products.

In a second embodiment, a recombinant plant viral polynucleotide is provided as in the first embodiment except that the native coat protein coding sequence is placed adjacent one of the non-native coat protein subgenomic promoters instead of a non-native coat protein coding sequence.
In a third embodiment, a recombinant plant viral polynucleotide is provided in which the native coat protein gene is adjacent its subgenomic promoter and one or more non-native subgenomic promoters have been inserted into the viral polynucleotide. The inserted non-native subgenomic promoters are capable of transcribing or expressing adjacent genes in a plant host and are incapable of recombination with each other and with native subgenomic promoters. Non-native polynucleotide sequences may be inserted adjacent the non-native subgenomic plant viral promoters such that the sequences are transcribed or expressed in the host plant under control of the subgenomic promoters to produce the desired product.

In a fourth embodiment, a recombinant plant viral polynucleotide is provided as in the third embodiment except that the native coat protein coding sequence is replaced by a non-native coat protein coding sequence.

The viral vectors are encapsidated by the coat proteins encoded by the recombinant plant viral polynucleotide to produce a recombinant plant vims. The recombinant plant viral polynucleotide or recombinant plant vims is used to infect appropriate host plants. The recombinant plant viral polynucleotide is capable of replication in the host, systemic spread in the host, and transcription or expression of foreign gene(s) (exogenous polynucleotide) in the host to produce the desired protein.


In addition to the above, the polynucleotide of the present invention can also be introduced into a chloroplast genome thereby enabling chloroplast expression.

A technique for introducing exogenous polynucleotide sequences to the genome of the chloroplasts is known. This technique involves the following procedures. First, plant cells are chemically treated so as to reduce the number of chloroplasts per cell to about one. Then, the exogenous polynucleotide is introduced via particle bombardment
into the cells with the aim of introducing at least one exogenous polynucleotide molecule into the chloroplasts. The exogenous polynucleotides selected such that it is integratable into the chloroplast's genome via homologous recombination which is readily effected by enzymes inherent to the chloroplast. To this end, the exogenous polynucleotide includes, in addition to a gene of interest, at least one polynucleotide stretch which is derived from the chloroplast's genome. In addition, the exogenous polynucleotide includes a selectable marker, which serves by sequential selection procedures to ascertain that all or substantially all of the copies of the chloroplast genomes following such selection will include the exogenous polynucleotide. Further details relating to this technique are found in U.S. Pat. Nos. 4,945,050; and 5,693,507 which are incorporated herein by reference. A polypeptide can thus be produced by the protein expression system of the chloroplast and become integrated into the chloroplast's inner membrane.

According to some embodiments of the invention, the method further comprising growing the plant expressing the exogenous polynucleotide under the biotic or abiotic stress (e.g., drought, water deprivation or temperature stress).

Thus, the invention encompasses (transgenic) plants, parts thereof or plant cells, exogenously expressing the polynucleotide(s) or the nucleic acid constructs of the invention.

Once expressed within the plant cell or the entire plant, the level of the polypeptide encoded by the exogenous polynucleotide can be determined by methods well known in the art such as, activity assays, Western blots using antibodies capable of specifically binding the polypeptide, Enzyme-Linked Immuno Sorbent Assay (ELISA), radio-immuno-assays (RIA), immunohistochemistry, immunocytochemistry, immunofluorescence and the like.

Methods of determining the level in the plant of the RNA transcribed from the exogenous polynucleotide are well known in the art and include, for example, Northern blot analysis, reverse transcription polymerase chain reaction (RT-PCR) analysis (including quantitative, semi-quantitative or real-time RT-PCR) and RNA-Z/z situ hybridization.

The effect of the expressed HXK on plant stomata conductance (e.g., manifested by aperture), water use efficiency, water use efficiency and/or photosynthesis can be
qualified using methods which are well known in the art. Stomata functionality assays are described in length in the Examples section which follows.

The effect of the exogenous polynucleotide encoding the HXK on abiotic stress tolerance can be determined using known methods such as detailed below and in the Examples section which follows.

**Abiotic stress tolerance** - Transformed (i.e., expressing the HXK) and non-transformed (wild type) plants are exposed to biotic or an abiotic stress condition, such as water deprivation or suboptimal temperature (low temperature, high temperature).

**Cold stress tolerance** - To analyze cold stress, mature (25 day old) plants are transferred to 4 °C chambers for 1 or 2 weeks, with constitutive light. Later on plants are moved back to greenhouse. Two weeks later damages from chilling period, resulting in growth retardation and other phenotypes, are compared between both control and transgenic plants, by measuring plant weight (wet and dry), and by comparing growth rates measured as time to flowering, plant size, yield, and the like.

**Heat stress tolerance** - Heat stress tolerance is achieved by exposing the plants to temperatures above 34 °C for a certain period. Plant tolerance is examined after transferring the plants back to 22 °C for recovery and evaluation after 5 days relative to internal controls (non-transgenic plants) or plants not exposed to neither cold or heat stress.

**Water use efficiency** - can be determined as the biomass produced per unit transpiration. To analyze WUE, leaf relative water content can be measured in control and transgenic plants. Fresh weight (FW) is immediately recorded; then leaves are soaked for 8 hours in distilled water at room temperature in the dark, and the turgid weight (TW) is recorded. Total dry weight (DW) is recorded after drying the leaves at 60 °C to a constant weight. Relative water content (RWC) is calculated.

**Salinity tolerance assay** - Transgenic plants with tolerance to high salt concentrations are expected to exhibit better germination, seedling vigor or growth in high salt. Salt stress can be effected in many ways such as, for example, by irrigating the plants with a hyperosmotic solution, by cultivating the plants hydroponically in a hyperosmotic growth solution (e.g., Hoagland solution), or by culturing the plants in a hyperosmotic growth medium [e.g., 50 % Murashige-Skoog medium (MS medium)]. Since different plants vary considerably in their tolerance to salinity, the salt
concentration in the irrigation water, growth solution, or growth medium can be adjusted according to the specific characteristics of the specific plant cultivar or variety, so as to inflict a mild or moderate effect on the physiology and/or morphology of the plants (for guidelines as to appropriate concentration see, Bernstein and Kafkafi, Root Growth Under Salinity Stress In: Plant Roots, The Hidden Half 3rd ed. Waisel Y, Eshel A and Kafkafi U. (editors) Marcel Dekker Inc., New York, 2002, and reference therein).

For example, a salinity tolerance test can be performed by irrigating plants at different developmental stages with increasing concentrations of sodium chloride (for example 50 mM, 100 mM, 200 mM, 400 mM NaCl) applied from the bottom and from above to ensure even dispersal of salt. Following exposure to the stress condition the plants are frequently monitored until substantial physiological and/or morphological effects appear in wild type plants. Thus, the external phenotypic appearance, degree of wilting and overall success to reach maturity and yield progeny are compared between control and transgenic plants.

Quantitative parameters of tolerance measured include, but are not limited to, the average wet and dry weight, growth rate, leaf size, leaf coverage (overall leaf area), the weight of the seeds yielded, the average seed size and the number of seeds produced per plant. Transformed plants not exhibiting substantial physiological and/or morphological effects, or exhibiting higher biomass than wild-type plants, are identified as abiotic stress tolerant plants.

**Osmotic tolerance test** - Osmotic stress assays (including sodium chloride and mannitol assays) are conducted to determine if an osmotic stress phenotype was sodium chloride-specific or if it was a general osmotic stress related phenotype. Plants which are tolerant to osmotic stress may have more tolerance to drought and/or freezing. For salt and osmotic stress germination experiments, the medium is supplemented for example with 50 mM, 100 mM, 200 mM NaCl or 100 mM, 200 mM NaCl, 400 mM mannitol.

The effect of the transgene on plant's vigor, growth rate, biomass, yield and/or oil content can be determined using known methods.

**Plant vigor** - The plant vigor can be calculated by the increase in growth parameters such as leaf area, fiber length, rosette diameter, plant fresh weight and the like per time.
Growth rate - The growth rate can be measured using digital analysis of growing plants. For example, images of plants growing in greenhouse on plot basis can be captured every 3 days and the rosette area can be calculated by digital analysis. Rosette area growth is calculated using the difference of rosette area between days of sampling divided by the difference in days between samples.

As mentioned, the present teachings are also directed at downregulating HXK activity or expression in a guard cell specific manner. This is effected to increase plant dehydration where needed. For example when there is a need to accelerate defoliation prior or after harvesting such as in cotton and other crops, or for dehydration of leaves and stems for straw for instance.

Downregulation (gene silencing) of the transcription or translation product of an endogenous HXK in a guard-cell specific manner can be achieved by co-suppression, antisense suppression, RNA interference and ribozyme molecules under the above mentioned cis-acting regulatory element active specifically in a guard cell.

Thus, there is provided a plant expression construct comprising a nucleic acid sequence encoding a nucleic acid agent for silencing expression of a hexokinase, wherein expression of said nucleic acid agent is under a transcriptional control of a guard cell-specific cis-acting regulatory element (as described above).

Co-suppression (sense suppression) - Inhibition of the endogenous gene can be achieved by co-suppression, using an RNA molecule (or an expression vector encoding same) which is in the sense orientation with respect to the transcription direction of the endogenous gene. The polynucleotide used for co-suppression may correspond to all or part of the sequence encoding the endogenous polypeptide and/or to all or part of the 5' and/or 3' untranslated region of the endogenous transcript; it may also be an unpolyadenylated RNA; an RNA which lacks a 5' cap structure; or an RNA which contains an unslicable intron. In some embodiments, the polynucleotide used for co-suppression is designed to eliminate the start codon of the endogenous polynucleotide so that no protein product will be translated. Methods of co-suppression using a full-length cDNA sequence as well as a partial cDNA sequence are known in the art (see, for example, U.S. Pat. No. 5,231,020).

According to some embodiments of the invention, downregulation of the endogenous gene is performed using an amplicon expression vector which comprises a
plant virus-derived sequence that contains all or part of the target gene but generally not
all of the genes of the native vims. The viral sequences present in the transcription
product of the expression vector allow the transcription product to direct its own
replication. The transcripts produced by the amplicon may be either sense or antisense
relative to the target sequence [see for example, Angell and Baulcombe, (1997) EMBO
6,646,805, each of which is herein incorporated by reference].

Antisense suppression - Antisense suppression can be performed using an
antisense polynucleotide or an expression vector which is designed to express an RNA
molecule complementary to all or part of the messenger RNA (mRNA) encoding the
endogenous polypeptide and/or to all or part of the 5’ and/or 3’ untranslated region of
the endogenous gene. Over expression of the antisense RNA molecule can result in
reduced expression of the native (endogenous) gene. The antisense polynucleotide may
be fully complementary to the target sequence (i.e., 100 % identical to the complement
of the target sequence) or partially complementary to the target sequence (i.e., less than
100 % identical, e.g., less than 90 %, less than 80 % identical to the complement of the
target sequence). Antisense suppression may be used to inhibit the expression of
multiple proteins in the same plant (see e.g., U.S. Pat. No. 5,942,657). In addition,
portions of the antisense nucleotides may be used to disrupt the expression of the target
gene. Generally, sequences of at least about 50 nucleotides, at least about 100
nucleotides, at least about 200 nucleotides, at least about 300, at least about 400, at least
about 450, at least about 500, at least about 550, or greater may be used. Methods of
using antisense suppression to inhibit the expression of endogenous genes in plants are
Nos. 5,759,829 and 5,942,657, each of which is herein incorporated by reference.
Efficiency of antisense suppression may be increased by including a poly-dT region in
the expression cassette at a position 3’ to the antisense sequence and 5’ of the
polyadenylation signal [See, U.S. Patent Publication No. 20020048814, herein
incorporated by reference].

RNA interference - RNA interference can be achieved using a
polynucleotide, which can anneal to itself and form a double stranded RNA having a
stem-loop structure (also called hairpin structure), or using two polynucleotides, which form a double stranded RNA.

For hairpin RNA (hpRNA) interference, the expression vector is designed to express an RNA molecule that hybridizes to itself to form a hairpin structure that comprises a single-stranded loop region and a base-paired stem.


According to some embodiments of the invention, the sense sequence of the base-paired stem is from about 10 nucleotides to about 2,500 nucleotides in length, e.g., from about 10 nucleotides to about 500 nucleotides, e.g., from about 15 nucleotides to about 300 nucleotides, e.g., from about 20 nucleotides to about 100 nucleotides, e.g., or from about 25 nucleotides to about 100 nucleotides.

According to some embodiments of the invention, the antisense sequence of the base-paired stem may have a length that is shorter, the same as, or longer than the length of the corresponding sense sequence.

According to some embodiments of the invention, the loop portion of the hpRNA can be from about 10 nucleotides to about 500 nucleotides in length, for example from about 15 nucleotides to about 100 nucleotides, from about 20 nucleotides to about 300 nucleotides or from about 25 nucleotides to about 400 nucleotides in length.


For double-stranded RNA (dsRNA) interference, the sense and antisense RNA molecules can be expressed in the same cell from a single expression vector (which comprises sequences of both strands) or from two expression vectors (each comprising the sequence of one of the strands). Methods for using dsRNA interference to inhibit the expression of endogenous plant genes are described in Waterhouse, et al., (1998) Proc. Natl. Acad. Sci. USA 95:13959-13964; and WO 99/49029, WO 99/53050, WO 99/61631, and WO 00/49035; each of which is herein incorporated by reference.

According to some embodiments of the invention, RNA interference is effected using an expression vector designed to express an RNA molecule that is modeled on an endogenous micro RNAs (miRNA) gene. Micro RNAs (miRNAs) are regulatory agents consisting of about 22 ribonucleotides and highly efficient at inhibiting the expression of endogenous genes [Javier, et al., (2003) Nature 425:257-263]. The miRNA gene
encodes an RNA that forms a hairpin structure containing a 22-nucleotide sequence that is complementary to the endogenous target gene.

Thus, the present teachings provide for a transgenic plant or a part thereof comprising the plant expression construct as described herein as well as isolated plant cell or a plant cell culture comprising the plant expression construct as described herein.

The present teachings also relate to processed products produced from the plants, plant parts or plant cells of the present invention. Such processed products relate to food, animal feed, beverages, construction material, biofuel, biodiesel, oils, sauces, pastes, pastries, meal and the like.

It is expected that during the life of a patent maturing from this application many relevant hexokinases and guard cell specific cis-acting regulatory elements will be developed and the scope of the terms used herein are intended to include all such new technologies a priori.

As used herein the term "about" refers to ± 10%.

The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to".

The term "consisting of" means "including and limited to".

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as
from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases "ranging/ranges between" a first indicate number and a second indicate number and "ranging/ranges from" a first indicate number "to" a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, cellular and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for

EXAMPLE 1
MATERIALS AND METHODS

Plant material and growth conditions

Experiments were conducted using WT tomato (Solanum lycopersicum cv. MP-1), isogenic independent transgenic homozygous tomato lines expressing different levels of the Arabidopsis AtHXK1 (35S::AtHXK1) [as previously described by Dai et al. (1999)], isogenic transgenic homozygous lines with antisense suppression of the tomato LeHXK1,2&3 genes, isogenic transgenic homozygous lines expressing GFP or AtHXK1 under the control of the KST1 promoter, and the ABA-deficient mutant Sitiens (Dai et al., 1999) (S. lycopersicum cv. Ailsa Craig).

Independent antisense-HXK tomato lines, aHK1 and aHK2, were generated following transformation of MP-1 with an antisense construct of StHXK1 (X94302) expressed under the 35S promoter. The potato StHXK1 shares over 80% sequence
identity with *LeHXK1,2&3* and conferred suppression of *LeHXK1,2&3* (Figure 4A). Arabidopsis (Col.) and tomato (MP-1) lines that express GFP or *AtHXK1* specifically in guard cells (GCGFP and GCHXK lines, respectively) were generated following transformation with GFP or *AtHXK1* expressed under the *KST1* promoter (Muller-Rober et al., 1995). Independent transgenic homozygous lines for each construct were then identified. The tomato plants were grown in a temperature-controlled greenhouse under natural growth conditions and the Arabidopsis plants were grown in a walk-in growth chamber kept at 22 °C, with an 8-h light/16-h dark photoperiod.

**Stomatal measurements**

Stomatal aperture and density are determined using the rapid imprinting technique described by Geisler and Sack (2002). This approach allows to reliably score hundreds of stomata from each experiment, each of which is sampled at the same time. Light-bodied vinylpolysiloxane dental resin (Heraeus-Kulzer, Hanau, Germany) is attached to the abaxial leaf side and then removed as soon as it dries (1 min). The resin epidermal imprints are than covered with nail polish, which removed once it had dried out and serves as a mirror image of the resin imprint. The nail-polish imprints are put on glass cover slips and photographed under bright-field microscope. Stomata images are later analyzed to determine aperture size using the ImageJ software (www.rsb.info.nih.gov/ij/) fit-ellipse tool or any other software that can process and analyze images. A microscopic ruler is used for the size calibration. Additional information can be obtained from the software such as stomata width, length, area, perimeter etc.

To assess stomatal responses, leaflets are cut and immediately immerse in artificial xylem sap solution (AXS) (Wilkinson and Davies, 1997) containing 100 mM sucrose (Duchefa Biochemie) with or without 20 mM N-acetyl glucosamine (NAG, Sigma-Aldrich), 100 mM or 200 mM glucose (Duchefa Biochemie), 100 mM or 200 mM fructose (Sigma-Aldrich), 100 mM 2-deoxyglucose (Sigma-Aldrich), 10 mM or 100 mM mannose (Sigma-Aldrich), 100 mM sorbitol (Sigma-Aldrich) or 100 mM or 200 mM mannitol (Duchefa Biochemie). The sorbitol and mannitol treatments serve as non-metabolic osmotic controls. Imprints are taken 3 h after immersion and stomatal aperture is analyzed. Different plant species can be used as well as, AXS solutions, treatment solutions and different timings to our decision.
Gas exchange analysis

Gas exchange measurements are assayed using a Li-6400 portable gas-exchange system (LI-COR). Plants are growing under favorable or stressed conditions, and measurements are conducted on fully expanded leaf, 5th-6th from top in the case of tomato. All measurements are conducted between 10:00 AM and 2:00 PM. We are inducing photosynthesis under saturating light (1000-1200 µmol m⁻² sec⁻¹) with 370 µmol m⁻² s⁻¹ CO₂ surrounding the leaf (Ca). The amount of blue light is set to 15% photosynthetically active photon flux density to optimize stomatal aperture. The leaf-to-air VPD (Vapor pressure deficit) is kept at around 1 to 2.5 kPa and leaf temperature is kept at around 25°C, during all measurements. Once a steady state is reached, measurements are done. It is possible to tune each of the above mentioned parameters. Each measurement contains data of photosynthesis (µmol CO₂ m⁻² s⁻¹), transpiration (mmol H₂O m⁻² s⁻¹), Stomatal conductance (mol H₂O m⁻² s⁻¹), and calculated instantaneous water use efficiency (µmol CO₂ mmol⁻¹ H₂O). Additional data obtained from each measurement are mesophyll conductance for CO₂ (mol CO₂ m⁻² s⁻¹ bar⁻¹), electron transport rate, calculated from PS (photosystem) II quantum yield and internal CO₂ concentrations (Ci).

For stomatal conductance (gₛ) measurements the leaf conductance steady-state porometer LI-1600 (LI-COR, Lincoln, NE) is used according to manufacture instructions.

Whole-plant transpiration measurements

Whole-plant transpiration rates and relative daily transpiration (RDT) are determined using a wide-screen lysimeter-scale system, which allows measurements of up to 160 plants simultaneously. Plants are planted in 3.9-L pots and grow under controlled conditions. Each pot is placed on a temperature-compensated load cell with digital output and is sealed to prevent evaporation from the surface of the growth medium. A wet vertical wick made of 0.15 m² cotton fibers partially submerged in a 1-L water tank is placed on a similar load cell and use as a reference for the temporal variations in the potential transpiration rate. The output of the load cells is monitored every 10 s and the average readings over 3 min are logged in a data logger for further analysis. The output data includes whole plant transpiration, plant weight, light intensity, vapor pressure deficit (VPD), temperature, stomatal conductance, water use
efficiency and additional environmental and physiological parameters. The whole-plant transpiration rate is calculated by a numerical derivative of the load cell output following a data-smoothing process (Sade et al., 2010). The plant's daily transpiration rate is normalized to the total plant weight and the data for neighboring submerged wick and these figures are averaged for a given line over all plants (amount taken up by the wick daily = 100%). Water use efficiency is calculated from the daily weight added against the daily water loss for each plant. Plants RDT is monitored under different growth conditions to our decision: Normal irrigation, drought, salt treatment and more. It is possible to shift growth conditions on a daily bases and to monitor plants responses.

RNA extraction, cDNA generation and quantitative real-time PCR expression analysis (Based on Goren 2011, Kandel-Kfir 2006)

Tissue samples are snap-frozen and homogenize in liquid nitrogen. RNA is extracted using the EZ-RNA kit (Biological Industries, Kibbutz Bet Haemek, Israel), with up to 500 μl of frozen homogenized tissue per extraction tube. At least four independent extractions are performed for each tissue set. The extractions are earned out according to the manufacturer’s protocol, including two optional washes in 2 M LiCl. RNA pellets are then suspended in 25 μl DEPC-treated H2O and treated with DNase (Ambion, Austin, TX, USA) according to the manufacturer’s instructions. RNA presence is confirmed by gel electrophoresis and DNA degradation is confirmed by PCR. RNA (≤1μg) from each sample is than reverse-transcribed to cDNA using MMLV RT (ProMega, Madison, WI, USA) in a 25-μl reaction, with 2 μl random primers and 1μl mixed poly-dT primers (18-23 nt). All cDNA samples are diluted 1:8 in DEPC-treated water.

Real-time reactions are prepared using SYBR Green mix (Eurogentec S.A., Seraing, Belgium) in 10 μl volumes with 4 μl diluted cDNA per reaction, two replicates per cDNA sample. Reactions run in a RotorGene 6000 cycler (Corbett, Mortlake, New South Wales, Australia), 40 cycles per run, with sampling after each cycle. Following an initial pre-heating step at 95°C for 15 min, there are 40 cycles of amplification consisting of 10s at 95°C, 15 s at 55°C, 10 s at 60°C and 20 s at 72°C. Results are than interpreted using RotorGene software, two duplicates per sample. Data are normalized using SICyP as a reference gene (cyclophilin - accession no. M55019). Primers used for amplification: SICyP - CGTCGTGTTTGGACAAGTTG (SEQ ID NO: 1) and
CCGCAGTCAGCAATAACCA (SEQ ID NO: 2). The primers for SIHXKs (LeHXKs) are as follows: for SIHXK1- GACTTGCTGGGAGAGGAGT (SEQ ID NO: 3) and AAGGTACATTGAATGAGAGGAGCA (SEQ ID NO: 4); for SIHXK2-GTCTCTCCCCATCTTTCCCTTG (SEQ ID NO: 5) and CCCAAGTACATACCAGAACAT (SEQ ID NO: 6); for SIHXK3-GCGATATTACACTCCCTCGTG (SEQ ID NO: 7) and CTGCTTCTCTCCGTCTTTAAA (SEQ ID NO: 8); and for SIHXK4-GCTGAGGACACCTGATATG (SEQ ID NO: 9) and GATCGGATTTTACCCAGCTA (SEQ ID NO: 10).

**Protein Extraction and analysis of Hexokinase activity**

Protein extraction from plant leaves is performed with 1 to 2 g of plant tissue homogenized in 4 volumes of extraction buffer (50 mM Hepes, pH 7.6, 1 mM EDTA, 15 mM KC1, 1 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 3 mM diethylidithiocabamic acid, and 0.2% PVP). The mixture is centrifuged for 25 min at 16,000g at 48C, and the supernatant is brought to 80% ammonium sulfate saturation. After centrifugation, the pellet is resuspended in 0.5 mL of washing buffer (50 mM Hepes, pH 7.5, 1 mM EDTA, and 1 mM DTT), desalted on a G-25 Sephadex column (55 x 11 mm), and used as a crude enzyme extract for subsequent enzymatic analysis.

Hexokinase activity is measured by enzyme-linked assay according to Schaffer and Petreikov (1997). The assays contain a total volume of 1 mL of 30 mM Hepes-NaOH, pH 7.5, 2 mM MgCl2, 0.6 mM EDTA, 9 mM KC1, 1 mM NAD, 1 mM ATP, and 1 unit of NAD-dependent glucose-6-phosphate dehydrogenase (G6PDH from *Leuconostoc mesenteroides*; Sigma). To assay glucose phosphorylation, the reaction is initiated with 2 mM glucose. Reactions are conducted at 37°C, and absorbion at 340 nm is monitored continuously. (For additional information see Dai et al. 1999, Schaffer and Petreikov, 1997).

**Monitoring nitric oxide production in guard cells**

Detection of nitric oxide (NO) levels in stomata is performed as follows: Epidermal peels are prepared and incubated in MES buffer [25 mM MES-KOH, pH = 6.15 and 10 mM KC1 (MES, 2-(N-morpholino)-ethane sulfonic acid; Sigma-Aldrich) with or without 20 mM NAG, for 2.5 h under steady light, and then loaded with 60 µM NO indicator dye, DAF-2DA (4, 5-diaminofluorescein diacetate; Sigma-Aldrich),
diluted in MES buffer with or without 20 mM N-acetyl glucosamine (NAG, Sigma-Aldrich) and left for an additional 50 min. Then, the peels are washed with MES 3 times and re-incubated for 30 min in the buffer (control, set as 100% fluorescence) or in 100 mM sorbitol, 100 mM sucrose and 20 mM NAG. The peels are then photographed under a microscope (see Materials and Methods, "Confocal microscopy imaging"). Three to four biological repeats containing 20-30 stomata each are included in each experiment and each experiment is repeated several times. Images are analyzed using the ImageJ software histogram tool to evaluate fluorescence intensity and the fit-ellipse tool to determine stomatal aperture. It is possible to use epidermal strips from different species, use different treatments solutions and different timings, all to our decision.

Confocal microscopy imaging

Images are acquired using the OLYMPUS IX 81 (Japan) inverted laser scanning confocal microscope (FLUOVIEW 500) equipped with a 488-nm argon ion laser and a 60X1.0 NA PlanApo water immersion objective. Nitric oxide- DAF-2DA (4, 5-diaminofluorescein diacetate; Sigma-Aldrich) fluorescence is excited by 488-nm light and the emission is collected using a BA 505-525 filter. GFP is excited by 488-nm light and the emission is collected using a BA 505-525 filter. A BA 660 IF emission filter is used to observe chlorophyll autofluorescence. Confocal optical sections are obtained at 0.5-µm increments. The images are color-coded green for GFP and magenta for chlorophyll autofluorescence.

Thermal imaging

Leaf temperature is a reliable tool for determine transpiration variation among different conditions and different plant species. High temperatures are associated with closed stomata and low transpiration, while low temperature points out for open stomata and high transpiration. For thermal imaging, leaves are imaged using a thermal camera (ThermaCAM model SC655; FLIR Systems). Pictures are later analyzed using the ThermaCAM researcher pro 2.10 software. The experiments are repeated several times. Data are means ± SE from five biological repeats per line; four leaves are analyzed per plant.

Use of KST1 as a guard cell specific promoter

The KST1 potassium channel in potato (Solanum tuberosum L.) has been shown to be expressed specifically in guard cells (Muller-Rober et al., 1995). Later, by GUS
activity and staining assay it has been demonstrated that KST1 promoter segment can be used to express genes exclusively in guard cells (Plesch et al., 2001). Using this knowledge, transgenic tomato and Arabidopsis plants were generated overexpressing Arabidopsis hexokinase \( KST::AtHXKl \) or GFP (green fluorescence protein) (as a control for exclusive expression) specifically in guard cells in the following procedures:

1. Creation of binary vector containing an insert of \( AtHXKl \) cDNA under KST1 promoter followed by terminator.
2. Creation of binary vector containing an insert of \( GFP \) gene under KST1 promoter followed by terminator.
3. Plant transformation.
4. Identification of plants containing \( KSTl::AtHXKl \) trait.

**Creation of a binary vector containing an insert of \( AtHXKl \) cDNA or GFP under KST1 promoter followed by terminator.**

The binary vector pGreen0029 was used (Hellens et al., 2000b) for transformation into tomato and Arabidopsis plants. The KST1 promoter was ligated upstream the \( AtHXKl \) coding sequence (isolated by (Dai et al., 1995) or GFP followed by a terminator (See Figures 17A-B).

**EXAMPLE 2**

**SUCROSE STIMULATES STOMATAL CLOSURE**

To examine the effect of Sue on stomata, intact wild-type (WT) tomato leaflets were immersed in artificial apoplastic solutions (Wilkinson and Davies, 1997) containing either 100 mM Sue or 100 mM sorbitol, a non-metabolic sugar used as an osmotic control, and measured stomatal aperture. Sue decreased stomatal aperture size by 29% relative to sorbitol (Figures 1A, B). Sucrose is a disaccharide that has to be cleaved. It may be cleaved by cell wall (apoplastic) invertases, yielding glucose (Glc) and fructose (Fru) in equal proportions (Granot, 2007) and resulting in additional extracellular osmolalities approaching 200 mOsm/L, as compared to the 100 mOsm/L of the original Sue added. We, therefore, compared the effects of 100 mM sucrose, 100 mM Glc + 100 mM Fru and 200 mM Glc or Fru with the effect of 200 mM mannitol, which was used as an additional osmotic control. All of the sugar combinations decreased the size of stomatal apertures, as compared to the effect of 200 mM mannitol.
(Figure 1C), supporting an osmotic-independent role for sugars in the regulation of stomatal closure.

EXAMPLE 3

SUCROSE STIMULATES STOMATAL CLOSURE VIA HEXOKINASE

Sucrose may be cleaved by either apoplastic (extracellular) invertase or enter the cells via sucrose transporters and then be cleaved by intracellular sucrose-cleaving enzymes to yield the hexoses Glc and Fru. The hexoses Glc and Fru must be phosphorylated by hexose-phosphorylating enzymes (Granot, 2007). In plants, hexokinases (HXK) are the only enzymes that can phosphorylate Glc and may also phosphorylate Fm (Granot, 2007, 2008). HXKs are intracellular enzymes known to play both kinetic and sugar-signaling roles (Rolland et al., 2006). To examine whether Sue stimulates stomatal closure via HXK, the effect of Sue was tested in the presence of N-acetyl glucosamine (NAG), an efficient inhibitor of HXK activity (Hofmann and Roitsch, 2000). NAG almost completely abolished the effect of Sue and prevented stomatal closure, supporting a role for HXK in the regulation of stomatal closure (Figure 1B).

EXAMPLE 4

INCREASED EXPRESSION OF HXK ENHANCES STOMATAL CLOSURE

To further explore whether HXK mediates stomatal closure, the effect of Sue was examined on well-characterized transgenic tomato plants expressing the Arabidopsis HXK1 (AtHXK1) under the control of the global non-specific 35S promoter (Dai et al., 1999). The stomatal aperture of AtHXK1-expressing plants (the HK4 line, which has a level of HXK activity that is 5 times higher than that of WT plants) was reduced by 21% relative to the control plants even under the control conditions (100 mM sorbitol) (Figure 1B), indicating that increased expression of HXK induces stomatal closure. The addition of Sue caused the stomata to close even further (Figure 1B) and the HXK inhibitor NAG abolished the closing effect of Sue, further supporting a role for HXK in the regulation of stomatal closure (Figure 1B).
EXAMPLE 5
DIRECT CORRELATION BETWEEN HXK ACTIVITY, STOMATAL CLOSURE AND REDUCED TRANSPIRATION

To examine the effect of HXK on tomato stomata, the stomatal apertures and conductance of tomato lines expressing increasing levels of AtHXKl were measured. (The HK37, HK4 and HK38 lines have levels of HXK activity that are 2, 5 and 6 times higher than those of WT plants, respectively) (Dai et al., 1999). The stomatal densities of the AtHXKl-expression lines are similar to those of WT plants (Table SI), yet both stomatal aperture and conductance were significantly reduced, in direct correlation with the level of AtHXKl expression (Figures 2A, 2B). Furthermore, continued measurement of transpiration over the course of the day revealed that AtHXKl lowered the transpiration rate per unit leaf area in the AtHXKl-expression lines, in correlation with the level of AtHXKl expression (Figure 2C), so that the cumulative whole-plant relative daily transpiration per unit leaf area (RDT) was clearly negatively correlated with HXK activity (Figure 2D).

To rule out the possibility that the observed decrease in transpiration was the result of inhibitory effects of AtHXKl on root water uptake or stem water transport, reciprocal grafting experiments were performed. HK4 shoots were grafted onto WT roots and WT shoots were grafted onto HK4 roots (Figure 3A). Continued measurements of the transpiration rates and cumulative whole-plant relative daily transpiration per unit leaf area of the grafted plants indicated that decreased transpiration was generally associated with HK4 shoots, with the roots having only minor influence (Figures 3B, 3C). To further examine the effect of HK4 stems on transpiration, triple-grafted plants were generated in which HK4 interstock replaced a portion of the stem of WT plants (Figure 3D). The HK4 interstock had no effect on RDT (Figure 3E), indicating that the decreased transpiration of AtHXKl-expressing plants was the result of reduced transpiration by the leaves and not reduced water uptake by the roots or attenuated transport through the stem. The effect of AtHXKl on leaf transpiration further indicates that HXK controls stomatal behavior that affects the transpiration of intact whole plants.
EXAMPLE 6
SUPPRESSION OF HXK INHIBITS STOMATAL CLOSURE

The role of HXK in stomatal closure was further examined using tomato and Arabidopsis plants with antisense suppression and knockdown mutants of HXK, respectively. Four HXKs are known in tomato plants, three of which (LeHXKl,2 and 3) are mitochondria-associated HXKs similar to the sugar sensor AtHXKI (Granot, 2007, 2008). Unlike the stomatal closure observed in tomato plants expressing high level of AtHXKI (Figures 2A, B), stomatal closure in tomato lines (aHKl and aHK2) with antisense suppression of LeHXKl,2&3 (Figure 4A) was diminished in response to Sue treatments (Figure 4B). Similarly, the Arabidopsis AtHXKI-knockout gin2-l mutant had higher stomatal conductance and a higher transpiration rate, as compared to wild-type control plants (Figures 8E, F), supporting the hypothesis that HXK plays a role in the regulation of stomatal closure.

EXAMPLE 7
HXK mediates stomatal closure independent of downstream metabolism of the phosphorylated sugars

To examine whether downstream metabolism of the phosphorylated sugars is required for stomatal closure, the effects of mannose (a glucose epimer at the second carbon atom) and 2-dexoxyglucose (2-dG - a glucose analog) were tested. Both of these sugars are phosphorylated by HXK, but 2-dG is not further metabolized and mannose is poorly metabolized (Klein and Stitt, 1998; Pego et al., 1999). Both mannose and 2-dG reduced stomatal aperture (Figure 5). A lower concentration of mannose (10 mM) also reduced stomatal aperture more than 100 mM glucose (Figure 5), in line with previous observations that mannose is more potent than glucose with regard to HXK-mediated sugar effects (Jang and Sheen, 1994; Pego et al., 1999). Moreover, the closure effect of 10 mM mannose further supports an osmotic-independent role of sugars in the stimulation of stomatal closure. The results with mannose and 2-dG suggest that HXK stimulates stomatal closure independent of downstream metabolism of the phosphorylated sugars.
EXAMPLE 8

SUCROSE STIMULATES AN ABA-SIGNALING PATHWAY IN GUARD CELLS

It has previously been shown that the sugar-signaling effects of HXK, such as the inhibition of photosynthesis and growth, are mediated by abscisic acid (ABA) [for an updated review see Rolland et al. (2006)], a well-known phytohormone that also induces stomatal closure. Therefore, it was speculated that Sue might modulate guard-cell aperture via the HXK and ABA within guard cells. ABA-signaling in guard cells is mediated by the rapid production of nitric oxide (NO), which is required for ABA-induced stomatal closure and serves as an indicator of stomatal-closure stimuli (Garcia-Mata et al., 2003; Neill et al., 2008). To examine the effect of Sue on the ABA-signaling pathway in guard cells, NO levels were monitored within guard cells in response to applications of Sue. Epidermal peels were incubated with Sue and monitored using the fluorescent NO indicator dye diaminofluorescein diacetate (DAF-2DA). Applications of 100 mM sorbitol had no effect on NO levels in guard cells (Figure 6A). However, the application of 100 mM Sue resulted in a 3.5-fold increase in guard-cell fluorescence, indicating a rapid increase in NO levels, which was correlated with stomatal closure (Figure 6A). The guard cells of untreated HK4 (AtHXKI-expressing line) epidermal peels exhibited high NO levels, similar to those of Suc-treated WT epidermal peels (Figure 6B), and the addition of Sue to the peeled HK4 epidermis led to even more intense fluorescence (Figure 6B).

To further examine the involvement of HXK in the production of NO in guard cells, the HXK inhibitor NAG was used with epidermal peels. NAG not only inhibited the effect of Sue and blocked stomatal closure (Figure 1B), it also prevented the production of NO (Figure 6C). Washing out NAG with 100 mM Sue led to the resumption of NO production within less than 30 min (Figures 6D, E). These results suggest that Sue elicits a guard cell-specific NO response via HXK.

To verify that ABA is indeed required for the stomatal NO response to Sue, the same experiments were conducted with the ABA-deficient tomato mutant Sitiens, whose stomata are always open (Neill and Horgan, 1985). Unlike what was observed for the WT plants, treating Sitiens epidermal peels with 100 mM Sue did not result in any increase in fluorescence or stomatal closure, indicating that there was no production...
of NO (Figure 6F). However, treating *Sitiens* peels with externally supplied ABA did trigger the production of NO (Figure 6F) and stomatal closure. These findings indicate that *Sitiens*’s guard cells retain their ability to respond to externally supplied ABA by producing NO and that only the absence of ABA production in the *Sitiens* mutant prevents Suc-triggered NO production and stomatal closure. This observation confirms that *Sitiens* stomata do not respond to Sue due to this mutant’s ABA deficiency and that ABA is a vital mediator of the stomatal response to Sue.

EXAMPLE 9
GUARD-CELL SPECIFIC EXPRESSION OF *ATHXK1* INDUCES STOMATAL CLOSURE AND REDUCES TRANSPIRATION OF TOMATO AND ARABIDOPSIS PLANTS.

To examine the role of HXK specifically in guard cells, tomato and Arabidopsis plants were generated that express *AtHXK1* under the *KST1* guard-cell specific promoter (Muller-Rober et al., 1995). The specific expression of the *KST1* promoter in tomato and Arabidopsis guard cells was verified by expression of GFP under the *KST1* promoter (GCGFP lines, Figures 7A-E). Expression of the *KST1* promoter was specific to guard cells in all of the examined plant organs and was not detected in organs that do not have stomata, such as roots (Figure 7E). Guard-cell specific expression was recorded from early seedling development, as observed in the hypocotyls of seedlings (Figure 7D), through the stages in which leaves are fully expanded (Figures 7A-C).

Unlike the expression of *AtHXK1* under the 35S promoter (Dai et al., 1999; Kelly et al., 2012), the expression of *AtHXK1* under the guard-cell specific *KST1* promoter (GCHXK lines) had almost no negative growth effect (Figures 8A, D). Yet, expression of *AtHXK1* under the *KST1* promoter reduced both stomatal conductance and transpiration in both tomato and Arabidopsis plants (Figures 8B, C, E, F). These results strongly support the hypothesized specific role of HXK in guard cells, regulating stomatal closure.
EXAMPLE 10

GFP EXPRESSION UNDER THE CONTROL OF THE FBPASE PROMOTER IS SPECIFIC TO MESOPHYLL CELLS

To discriminate between HXK effects in guard cells versus mesophyll cells the present inventors have created transgenic tomato and Arabidopsis plants expressing HXK under a mesophyll promoter FBPase (Peleg et al., 2007). The specific expression of FBPase promoter was demonstrated with transgenic tomato and Arabidopsis plants expressing GFP under control of this promoter (designated MCGFP, Figure 9). Several independent homozygous Arabidopsis and tomato lines with high expression of FBPase:AtHXK1 (named MCHXK plants) were identified.

EXAMPLE 11

ELEVATED EXPRESSION OF HEXOKINASE IN GUARD CELLS REDUCES WHOLE PLANT TRANSPIRATION AND INCREASES WATER USE EFFICIENCY, AS DETERMINED USING GAS EXCHANGE ANALYSIS SYSTEM

Using the LI-COR gas exchange system the present inventors have analyzed 10 GCHXK independent lines and discovered a striking increase in water use efficiency in those plants (Figures 10A-D). Our data clearly shows that while photosynthesis remained unchanged (Figure IOC), stomatal conductance (indicating stomatal aperture, Figure 10B) and transpiration (Figure 10A) were reduced by 20% and 15% respectively, thus improving water use efficiency from 1.36 in WT to 1.78 in GCHXK lines (Figure 10D).

EXAMPLE 12

ELEVATED EXPRESSION OF HEXOKINASE IN GUARD CELLS REDUCES WHOLE PLANT TRANSPIRATION AND INCREASES WATER USE EFFICIENCY, AS DETERMINED USING LYSIMETER SCALES SYSTEM

To evaluate water use efficiency in GCHXK plants the present inventors used the precise and sensitive lysimeter scales system, which measures plant weight accumulation and total plant water loss during long lasting experiments, and can monitor more than 160 plants simultaneously under varied irrigation treatments (Figures
Two independent GCHXK transgenic lines (that exhibited high WUE when measured by LI-COR (Figures A-D 10)) were analyzed. The present inventors have discovered that relative daily transpiration of these lines was lower than WT throughout the entire experiment (20 days) (Figures 11A-C). Plant weight accumulation and growth were not affected. As a result, there was about 20%-30% increase in WUE in GCHXK lines compare to WT plants.

EXAMPLE 13
ELEVATED EXPRESSION OF HEXOKINASE IN GUARD CELLS REDUCES WHOLE PLANT TRANSPERSION RATE AND STOMATAL CONDUCTANCE, WITHOUT ANY NEGATIVE EFFECT ON GROWTH, THUS ENHANCING WATER USE EFFICIENCY

Using lysimeter scales system we further analyzed water saving and WUE in GCHXK plants, which displayed high WUE when measured by LI-COR (Figures 10A-D) and by lysimeter (Figures 11A-C). Several parameters were monitored. Parameters for water loss: transpiration rate, stomatal conductance ($g_s$); parameters for growth: total plant weight, total plant leaf area and environmental parameters: light intensity, vapor pressure deficit (VPD). It was found that along the day, the transpiration rates normalized to total leaf area were correlated with environmental changes (light intensity and VPD, Figures 12E and 12F respectively). Transpiration rates of GCHXK plants were significantly lower compared with those of WT along the day (Figure 12A). Accordingly, stomatal conductance was found to be reduced as well (Figure 12B) proving that in GCHXK plants, water are saved and stomata are more closed. Moreover, by measuring total plant leaf area and weight (Figures 12C and 12D respectively), the present inventors discovered that even though plants have consumed less water (Figure 12A) growth was not impaired, and was even improved as in the case of GCHXK line. Saving water without affecting plant growth improves whole plant water use efficiency.

EXAMPLE 14
ELEVATED EXPRESSION OF HEXOKINASE IN GUARD CELLS ENHANCES DROUGHT TOLERANCE

To monitor plants behavior under stress conditions the lysimeter scales system was used. After irrigation was fully stopped, plants were exposed to drought stress, which
gradually increased each day throughout the experiment. Transpiration rates of WT and GCHXK plants were analyzed for nine consecutive days (Figure 13). During the first 3 days GCHXK plants transpired less than WT, in line with normal conditions behavior (Figures 11A-C; 12A-F), indicating stress was only moderate at that time. However, in the following days (4 and 5), a transition between WT and GCHXK transpiration rates was observed (Figure 13, *) and WT transpiration was steeply dropped compared with GCHXKs, indicating that WT plants are more sensitive to drought. As seen in moderate stress (days 5 and 6) as well as in severe stress conditions (days 7 and 8), GCHXK transpiration is less sensitive to water limitation compare to WT, displaying slower decline in transpiration throughout the experiment. These results indicate that GCHXK plants have better tolerance to water shortage and that under mild-stress conditions these plants can still function normally. Drought tolerance was also detected while monitoring relative daily transpiration (RDT) of WT and GCHXK plants under drought conditions (Figure 11A). While shifting from irrigated to drought conditions (Figure 11A, days 10-11, magnified), a steep reduction in transpiration was observed for WT plants (red arrow). However, GCHXK transpiration was only moderately affected when exposed to drought (green arrow), indicating that these plants have better tolerance to drought.

EXAMPLE 15
ELEVATED EXPRESSION OF HEXOKINASE IN GUARD CELLS IMPROVES YIELD PRODUCTION

To examine the effect of GCHXK on yield, fruits number of GCHXK plants was monitored. Neither of the lines exhibited reduced yield, even though transpiration of these lines was found to be lower (Figures 10-12). On the contrary, in few lines fruit number was even higher than control (Figures 14A-B).

EXAMPLE 16
ELEVATED EXPRESSION OF HEXOKINASE IN GUARD CELLS IMPROVES YIELD PRODUCTION UNDER LIMITED WATER SUPPLY CONDITIONS

For a wide-range yield production assay, plants were grown in a controlled semi-commercial greenhouse under four different water stressed-irrigation regimes.
Plants were irrigated either 25% above the recommended irrigation amount (125%), the recommended irrigation (100%) and deficit irrigation (75%, 50% irrigation regimes, Figure 15A). Fruits were collected and cumulative fmit numbers and total fmit weight of each plant were documented (Figures 15B-C). As clearly seen, GCHXK on yield was dramatic. Compare to WT, GCHXK plants had significantly higher yield (fiait number and total fmit weight under all irrigation regimes. Yet, deficit irrigation did not alter fmit number per plant but reduced fmit weight. Interestingly, GCHXK fmit weight under fully stressed conditions (50% irrigation) was higher than control plants at 100% irrigation. GCHXK plants have also better tolerance to water limitation. When lowering the irrigation from 100% to 75%, fmit weight of GCHXK plants was reduced by only 16% while that of WT control plants was reduced by 39%. Hence, in addition to more yield under normal (100%) irrigation conditions (Figures 14A-B and Figure 15B), GCHXK plants also have better tolerance (higher yield) to limited water supply. Together with the transpiration results (Figure 13), these results indicate that specific expression of HXK in guard cells saves water, increases water use efficiency and improves yield production, not only under normal, but also under drought conditions as well.

EXAMPLE 17

ELEVATED EXPRESSION OF HEXOKINASE IN GUARD CELLS REDUCES WHOLE PLANT TRANSPIRATION, INDUCES STOMATAL CLOSURE AND INCREASES WATER USE EFFICIENCY IN ARABIDOPSIS

Thermal imaging and gas-exchange analysis were used to determine stomatal aperture, transpiration and WUE in Arabidopsis plants expressing HXK specifically in guard cells (GCHXK, Figures 16A-F). The present inventors have discovered that in GCHXK plants, stomatal conductance and transpiration (Fig. 16A and B respectively, Fig. 8E-F) are significantly reduced compare to WT. Additionally, by using thermal imaging technique, it was found that the leaf temperature of GCHXK plants was higher than WT, which indicates that stomata are more closed (Figure 16F). In addition, while transpiration was reduced, photosynthesis rates (Figures 16C), as well as the mesophyll conductance to CO₂ (gm, Figure 16D) were not affected. Moreover, growth was not affected as well (Figure 8D). Overall, GCHXK plants had higher water use efficiency
These results demonstrate that the same transgenic insertion of hexokinase under guard-cell specific promoter used in the case of Tomato (Solanaceae family) is universally applicable while affecting stomata and increases water use efficiency in the case of Arabidopsis (Brassicaceae family) as well, and that this technique could be implemented in other species as well.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.
LITERATURE CITED


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WHAT IS CLAIMED IS:

1. A plant expression construct comprising a nucleic acid sequence encoding a hexokinase under a transcriptional control of a guard cell-specific cis-acting regulatory element.

2. A plant expression construct comprising a nucleic acid sequence encoding a nucleic acid agent for silencing expression of a hexokinase, wherein expression of said nucleic acid agent is under a transcriptional control of a guard cell-specific cis-acting regulatory element.

3. The plant expression construct of claim 1 or 2, wherein said guard cell-specific cis-acting regulatory element is inducible.

4. The plant expression construct of claim 1 or 2, wherein said guard cell-specific cis-acting regulatory element is constitutive.

5. The plant expression construct of claim 1 or 2, wherein said guard cell-specific cis-acting regulatory element is a guard-cell specific promoter.

6. The plant expression construct of claim 5, wherein said guard-cell specific promoter is KST1 promoter.

7. A method of regulating plant stomata conductance, the method comprising modulating in the plant the level and/or activity of a hexokinase in a guard cell specific manner, thereby regulating plant conductance.

8. The method of claim 7, wherein said modulating is upregulating.

9. The method of claim 8, wherein said upregulating is effected by introducing the nucleic acid construct of claim 1 into the plant.
10. The method of claim 7, wherein said modulating is downregulating.

11. The method of claim 9, wherein said downregulating is effected by introducing into the plant a nucleic acid silencing agent under a transcriptional control of a guard cell-specific cis-acting regulatory element.

12. A method of decreasing plant stomata conductance, the method comprising introducing into a cell of a plant the nucleic acid constiaict of claim 1, thereby decreasing the stomata conductance of the plant.

13. A method of increasing water use efficiency of a plant, the method comprising introducing into a cell of the plant the nucleic acid constiaict of claim 1, thereby increasing water use efficiency of the plant.

14. A method of increasing tolerance of a plant to drought, salinity or temperature stress, the method comprising introducing into a cell of the plant the nucleic acid constiaict of claim 1, thereby increasing tolerance of the plant to drought, salinity or temperature stress.

15. A method of increasing biomass, vigor or yield of a plant, the method comprising introducing into a cell of the plant the nucleic acid constiaict of claim 1, thereby increasing the biomass, vigor or yield of the plant.

16. A method of increasing tolerance of a plant to biotic stress, the method comprising introducing into a cell of the plant the nucleic acid constiaict of claim 1, thereby increasing tolerance of the plant to biotic stress.

17. A transgenic plant or a part thereof comprising the plant expression constiaict of any one of claims 1-8.

18. An isolated plant cell or a plant cell culture comprising the plant expression constiaict of any one of claims 1-8.
19. The transgenic plant of claim 17 or the isolated plant cell or plant cell culture of claim 18, comprising the plant expression construct of claim 1, 7 or 8.

20. The part of the transgenic plant of claim 17, being a seed.

21. The part of the transgenic plant of claim 17, being a leaf.

22. The part of the transgenic plant of claim 17, wherein said seed is a hybrid seed.

23. The method of any one of claims 12-15, further comprising growing the plant under water deficient conditions.

24. The method of any one of claims 12-15, further comprising growing the plant under salinity.
FIG. 5
FIG. 9

MCGFP

Tomato  Arabidopsis

10mm  10mm
A. CLASSIFICATION OF SUBJECT MATTER

IPC (2013.01) C12N 15/82, A01H 5/10, A01H 5/12, C12N 15/113, C12N 15/54, A01H 5/02

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)

IPC (2013.01) C12N 15/82, A01H 5/10, A01H 5/12, C12N 15/113, C12N 15/54, A01H 5/02

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

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

Databases consulted: THOMSON INNOVATION, CAPLUS, BIOSIS, EMBASE, MEDLINE, Google Scholar

Search terms used: (Hexokinase expression or hexokinase downregulation or hexokinase silencing) and (plant vector or transgenic plant) and KST1 promoter and plant stress and (yield or biomass or vigor)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Category</th>
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<td>page 342 (right column), pages 344-345</td>
<td>14,16,23,24</td>
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<td>WO 20071 16394 A2 STATE OF ISRAEL, MINISTRY OF AGRICULTURE &amp; RURAL DEVELOPMENT, AGRICULTURAL RESEARCH ORGANIZATION; GRANOT, DAVID) 18 Oct 2007 (2007/10/18) claims 2, 5-8, 12</td>
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[X] Further documents are listed in the continuation of Box C.  [X] See patent family annex.

* Special categories of cited documents:
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Date of the actual completion of the international search: 05 Feb 2013
Date of mailing of the international search report: 05 Feb 2013

Name and mailing address of the EOA: Israel Patent Office
The Technology Park, Bldg.5, Malcha, Jerusalem, 9695 101, Facsimile No. 972-2-5651616

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MAZEL Alexander
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