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(54) Title: THERMOSTABLE RUBISCO ACTIVASE COMPLEXES

(57) Abrégé/Abstract:
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(57) **Abstract**: The present invention relates to Rubisco activase (RCA) and functionally equivalent polypeptides that confer thermo - stability to a complex comprising Rubisco activase and Ribulose-1,5-bis-phosphate carboxylase/oxygenase (Rubisco).

THERMOSTABLE RUBISCO ACTIVASE COMPLEXES

Technical Field

[001] The present invention relates to thermostable complexes comprising Rubisco activase. In particular the invention relates to thermostable complexes comprising Rubisco activase and Ribulose-1,5-bis-phosphate carboxylase/oxygenase and their uses for heat-tolerant plants.

Background

[002] During photosynthesis plants generate biomass by converting inorganic carbon in the form of CO_2 to carbohydrates using light energy. Accordingly, increasing the efficiency of photosynthesis is a target for improving crop productivity via conventional breeding practices and transgenic approaches.

[003] Ribulose-1,5-bis-phosphate carboxylase/oxygenase (Rubisco) catalyzes the incorporation (fixation) of CO_2 into ribulose-1,5-bisphosphate to yield two molecules of 3-phosphoglycerate. Rubisco can be present in either an active or inactive state and the conversion of the inactive state to the active state by rubisco activase is required for CO_2 fixation. This process entails the carbamylation of a specific lysine residue of Rubisco. When ribulose-1,5-bisphosphate from the stroma attaches to Rubisco it causes conformational changes, allowing the enzyme to catalyse the reaction of CO_2 with the newly sequestered sugar bisphosphate and starting a catalytic cycle. However, in the absence of Rubisco activase (RCA), the tight binding of ribulose-1,5-bisphosphate to the uncarbamylated Rubisco inactivates Rubisco so the rate of CO_2 fixation declines. RCA uncouples ribulose-1,5-bisphosphate from decarbamylated active sites and thus promotes the access of CO_2 for the carbamylation (activation) of the enzyme. That is, the activation of Rubisco enhances its catalytic capacity and thus facilitates photosynthesis.

[004] RCA is generally present in two forms, termed alpha (long form) and beta (short form), with the latter being a truncated form of the alpha protein.

[005] Photosynthesis is reduced when the temperature exceeds the optimum range for a particular plant and the reduction in photosynthesis at elevated temperatures is linked to Rubisco deactivation due to the inhibition of RCA.

[006] The present invention is based on the finding that complexes comprising *Oryza australiensis* RCA and Rubisco are active at elevated temperatures.

Disclosure of Invention

[007] The present inventors have found that the Rubisco activase (RCA) from *Oryza australiensis* confers thermostability to a complex of RCA with endogenous Ribulose-1,5-bis-phosphate carboxylase/oxygenase (Rubisco). It has also been found that the isolated RCA from *Oryza australiensis* confers thermostability to a complex formed with Rubisco from other plants. Accordingly, the isolated RCA from *Oryza australiensis* can be used to enhance heat tolerance in a plant.

[008] In a first aspect there is provided a complex comprising RCA from *Oryza australiensis,* or functional variants thereof, and a Rubisco that is not from *O. australiensis* wherein the RCA confers thermostability to the complex.

[009] The RCA may comprise at least one of SEQ ID NOs: 2 to 37, 39 or 40.

[010] In a second aspect, there is provided a RCA from *Oryza australiensis*, and functional variants thereof, capable of forming a complex with a Rubisco from a plant other than *O. australiensis and* conferring thermostability to the complex.

[011] In some embodiments the RCA comprises at least one of SEQ ID NOs: 2 to 37, 39 or 40. In some embodiments the RCA has at least 90%, at least 95%, at least 99% or 100% sequence identity to SEQ ID NO: 39 or 40.

[012] In a third aspect there is provided an isolated nucleic acid encoding the thermostable RCA of the second aspect of the present invention.

[013] In some embodiments the isolated nucleic acid encoding thermostable RCA comprises at least one of SEQ ID NOs: 41, 42, 50, 51 and 52. In some embodiments the isolated nucleic acid has at least 90%, at least 95%, at least 99% or 100% sequence identity to SEQ ID NOs: 41, 42, 50, 51 and 52.

[014] In an embodiment the isolated nucleic acid comprises SEQ ID NO: 51.

[015] In a fourth aspect, there is provided a chimeric gene or vector comprising the nucleic acid of the third aspect of the present invention.

[016] The nucleic acid is operably linked to a promoter, typically a plant expressible promoter. The promoter may be a heat-inducible promoter, green-tissue promoter or a chemical-inducible promoter.

[017] In a fifth aspect there is provided a host cell comprising the complex of the first aspect of the present invention, the RCA of the second aspect of the present invention, the nucleic acid of the third aspect of the present invention, or the vector of the fourth aspect of the present invention.

[018] In an embodiment the host cell is a plant cell. In an embodiment the plant cell is a rice plant cell.

[019] In a sixth aspect, there is provided a thermostable complex of the first aspect of the present invention in a plant species other than *O. australiensis* to enhance heat tolerance in the plant.

[020] In one embodiment, the plant species is of the genus *Oryza*. In some embodiments the plant or plant cell is not *O. australiensis.*

[021] In some embodiments the plants are selected from barley, maize, tomato, potato, rice, soybean, cotton, sunflower, alfalfa, lettuce, canola (oilseed rape), sorghum, and wheat.

[022] In an embodiment the plant is rice. In another embodiment, the plant is wheat.

[023] In a seventh aspect there is provided a method of enhancing heat tolerance in a plant by expressing the RCA of the second aspect of the present invention in the plant.

[024] The method may further comprise providing the plant with a nucleic acid of the third aspect of the present invention or a chimeric gene or vector of the fourth aspect of the present invention.

[025] In one embodiment the plant is capable of fixing carbon dioxide in heat stress conditions.

[026] In one embodiment the RCA is expressed in the one or more plastids of the plant.

[027] In another embodiment the expression of the RCA results in an increase in the rate of photosynthesis in the plant under heat stress conditions.

[028] In an eighth aspect there is provided use of a complex comprising a Rubisco activase (RCA) from *Oryza australiensis* and a Rubisco from a plant other than *O. australiensis* to confer thermostability to the complex in a plant by the RCA.

[029] Throughout this specification, unless the context requires otherwise, the word 'comprise', or variations such as 'comprises' or 'comprising', will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

[030] Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this specification.

[031] Some *Oryza* species are identified herein using the post-script cy, Cy, or CY which refers to Cape York, Australia which is the origin of the accession. Similarly other *Oryza* species are identified with using the post script; kr, or KR which refers to Keep River, Australia, the origin of the accession.

[032] In order that the present invention may be more clearly understood, embodiments will be described with reference to the following drawings and examples.

Brief Description of the Drawings

[033] **Figure 1.** Effect of high temperature on the leaf elongation rate (LER) of rice genotypes. Elongation rates were measured over a 12-h period for O. sativa cv. Doongara (closed circles), *O. meridionalis*-CY (closed squares) and *O. meridionalis*-KR (open squares) and *O. australiensis* (closed triangles). Values are expressed as a percentage of the leaf elongation rate (LER) of *O. sativa* cv. Amaroo, measured over the same period. After 4 h at 28°C, the temperature was increased to 45°C, maintained for 4 h, and then reduced to 28°C, as indicated by the shaded (45°C) area. Measurements of leaf length commenced 1 h prior to the beginning and ended at the end of the 12-h photoperiod. The solid grey line is the ambient temperature of the cabinet. Values are the mean of six experimental replicates. For clarity error bars have been omitted.

[034] **Figure 2.** Leaf elongation rate (LER) of five rice genotypes over 4 h at 28 or 45°C. LER was measured at either 28°C (filled circles/solid line) or 45°C (open circles/dashed line) over a 4-h period for *O. sativa* cv. Amaroo (Os-A) (a); *O. sativa* cv. Doongara (Os-D) (b); *O. meridionalis*-CY (Om-CY) (c); *O. meridionalis*-KR (Om-KR) (d); and *O. australiensis* (e). In panel f, the LER at 45°C of all five genotypes are plotted together. Values are means ± SE (n = 6).

[035] **Figure 3.** The effect of elevated temperature on gas-exchange ain five rice genotypes as annotated in Figure 1. Net photosynthetic rate (P_n) was measured at 28°C (open bars) or at 45°C (closed bars). Plants were exposed to 45ºC for 2 to 4 h prior to measurement. Temperature-dependent differences in parameters for each genotype were determined using a two-sampled t-test. * indicates significance at $P < 0.05$, ** indicates significance at $P < 0.01$ and *** indicates significance at $P < 0.001$. Values are means ± SE of three pot replicates.

[036] **Figure 4.** The relationship between leaf elongation rate (LER) and Rubisco activation state in five rice genotypes (*O. sativa* cvs Amaroo and Doongara; *O. meridionalis* CY and KR; *O. australiensis*) at 28 and 45°C. The mean LER for each genotype at each

growth condition was plotted against the corresponding activation state and a linear regression analysis was performed (solid line, y=0.033x+1.45, P=0.0004, r^2=0.5106).

[037] **Figure 5.** Concentration-dependent Rubisco activation by leaf-extracted RCA from *Oryza sativa* (Os) (open circles) and *Oryza australiensis* (Oa) (open squares), as well as by recombinant RCA from Os (solid lines) and Oa (dashed lines). The two lines for each species represent the alpha and beta recombinant isoforms, with the beta isoform always more active and therefore the upper line. RCA concentration is given in mass per volume, molarity and the molar ratio of RCA to Rubisco within the assay. For all assays the Rubisco concentration used was 1.5 $mg.mL^{-1}$ (2.7 µM). Molarity was calculated using the molecular weight of the complete Rubisco complex (553 kDa) and active complex of RCA (220 kDa) based on oligomer formation (Keown, J. R., *et al.*, (2013). *J. Biol. Chem.* 288, 20607-20615.). The solid vertical line and the dashed vertical line represent the expected *in vivo* concentration of RCA from Os and Oa, respectively, given the Rubisco concentration used. All measurements were taken at 30°C.

[038] **Figure 6.** The influence of leaf-extracted proteins on the thermostability of recombinant Oa beta RCA (activity shown by filled squares). Values are the means ± SE of three experimental replicates. The open squares were derived by subtracting the blue-filled circles (leaf protein extract with RCA removed) from the filled triangles (recombinant Oa beta RCA incubated in leaf protein extract with RCA removed).

[039] **Figure 7.** Vector maps of plasmids containing RCA genes, adapted to *E. coli* codon usage, for expression in *E. coli* and the nucleotide sequence of the respective RCA genes. All RCA genes were cloned into pET-23d+. (**A)** rcaIOs-2Pb containing RCA long isoform from *Oryza sativa* SEQ ID NO: 46; (**B**) rcaIOau-2Pb containing RCA long isoform from *Oryza australiensis* SEQ ID NO: 47; (**C**) rcaIIOs-3Pb containing RCA short isoform from *Oryza sativa* SEQ ID NO: 48; (**D**) rcaIIOau-3Pb containing RCA short isoform from *Oryza australiensis* SEQ ID NO: 49; (**E**) rcaIIOau-2Pb containing RCA long form of Rubisco activase nucleic acid from *O. australiensis* (adapted to rice codon usage) SEQ ID NO: 50; (**F**) rcaIIOau-3Pb containing RCA short form of Rubisco activase nucleic acid from *O. australiensis* with a 5AA truncation at the end of the gene SEQ ID NO: 51; (**G**) rcaIIOau-3Pb containing RCA short form of Rubisco activase nucleic acid from *O. australiensis* (adapted to rice codon usage) SEQ ID NO: 52.

[040] **Figure 8.** ATP hydrolysis activity of the α (panel A) and β (panel B) recombinant RCA isoforms from *O. sativa* (filled circles/solid line) and *O. australiensis* (open circles/dashed line) calculated using the NADH coupled assay.

[041] **Figure 9.** (**A**): ATP hydrolysis activity of the α recombinant RCA isoforms from *O. sativa* (filled circles/solid line) and *O. australiensis* (open circles/dashed line) calculated

using the phosphate release assay. (**B**): ATP hydrolysis activity of the β recombinant RCA isoforms from *O. sativa* (filled circles/solid line) and *O. australiensis* (open circles/dashed line) calculated using the phosphate release assay.

[042] **Figure 10.** ATP hydrolysis activity of *O. sativa* (Os) or *O. australiensis* (Oa) recombinant RCA determined by the NADH coupled assay (open circles) or the phosphate release assay (closed circles) normalised to 28°C and 30°C, respectively. Note that both methods give a similar temperature response of ATP hydrolysis except for a displacement to higher temperatures when phosphate release is assayed.

[043] **Figure 11.** Temperature-dependent Rubisco activation activity, determined using the same assay as in Figure 13, for leaf-extracted RCA_{Os} (filled circles/solid lines) and leaf-extracted RCA_{Oa} (open circles/dashed lines). Activation rates are given as individual measurements of the three biological replicates over the temperature range with quadratic equations fitted (**A**), after differences in RCA protein concentration between the species were accounted for (**B**) and when protein adjusted values were normalised to values measured at 30°C (**C**).

[044] **Figure 12.** Rubisco activation activity of recombinant RCA measured at either 30ºC or 36°C. The two isoforms of RCA were measured in isolation or mixed in α to β ratio of 8:1, 4:1, 2:1, 1:1. Values are means SE of three experimental replicates.

[045] **Figure 13.** Temperature-dependent activation of Rubisco by RCA extracted from Os and Oa leaf extracts (LE) and measured at a concentration of 0.2 $mg.mL^{-1}$ for Os and varying concentrations of 0.24, 0.49 or 0.96 $mg.mL^{-1}$ for Oa (indicated by values next to corresponding curves). A constant 1.5 $mg.mL^{-1}$ Os Rubisco concentration was used in all assays. The top panel is absolute values and the bottom is values relativized to 30°C.

[046] **Figure 14.** Differential Scanning Fluorimetry (DSF) of the alpha and beta isoforms of recombinant *O. sativa* (solid lines) and *O. australiensis* (dashed lines) RCA. The first derivative of detected fluorescence (dR) is plotted against temperature and the maximum in dR corresponds to the melting temperature of the protein (T_m). One $mg.mL^{-1}$ of protein was used for all samples. Each curve is the mean of three sample replicates. The shaded area represents the range of T_m across the four variants of RCA, extending from 41 to 43°C.

[047] **Figure 15.** Heat-dependent response of gene expression for the alpha and beta isoforms of RCA (left) and heat-dependent protein abundance of the alpha and beta isoform of RCA generated from image analysis of anion-exchange purified leaf samples. The ratio of the alpha to beta isoform is presented as alpha divided by beta mRNA or alpha divided by beta protein.

[048] **Figure 16.** Effect of recombinant RCA concentration on activity of the alpha and beta forms of RCA as measured by ATPase activity for *O. sativa* (upper panel) and *O. australiensis* (lower panel). Values are means ± SE of three experimental replicates.

[049] **Figure 17.** Temperature-dependent stability of the self-aggregating recombinant RCA oligomer and RCA-Rubisco complex for *O. sativa* (top panel) and *O. australiensis* (bottom panel). Stability is defined as no substantial decline in activity relative to that at 30°C. Stability of the RCA oligomer is purported to equate to stability of ATP hydrolysis activity. Stability of the RCA-Rubisco complex is purported to represent stability of recombinant RCA-mediated Rubisco activation. Temperatures at which the RCA-Rubisco complex is heat-labile is light-grey shaded, whereas the heat-labile range for both the RCA-Rubisco complex and RCA oligomer are dark-grey shaded.

[050] **Figure 18.** Temperature-dependent stability of Rubisco activation by recombinant and leaf extracted RCA_{Os} and RCA_{Oa} in combination with *O. sativa* ($Rubisco_{Os}$) and *O. australiensis* ($Rubisco_{Oa}$) Rubisco. Leaf-extracted RCA_{Os} and RCA_{Oa} in combination with $Rubisco_{Oa}$ and recombinant RCA_{Os} and RCA_{Oa} in combination with $Rubisco_{Oa}$ is in duplicate.

[051] **Figure 19.** Heat-dependent gene response of the alpha and beta isoform of RCA. Image analysis of cDNA from three biological replicates, with one set given on the right, allowed for the ratio of alpha to beta isoform to be determined. The ratio of alpha to beta isoform is presented as the % of total RCA mRNA that is comprised of the alpha isoform.

[052] **Figure 20.** Treatment effect on RCA extracted from control, heat-treated and post-heat treatment leaves, as determined by comparisons of fraction-activated values at 38°C compared to 28°C.

[053] **Figure 21.** Temperature-dependent stability of the self-aggregating RCA oligomer (open circles), the recombinant RCA-Rubisco complex (closed circles) and the native RCA-Rubisco complex (open triangles) for *O. sativa* (top panel) and *O. australiensis* (bottom panel). The arrow indicates the dramatic improvement in heat-stability of the *O. australiensis* native RCA-Rubisco complex relative to the recombinant complex. Due to the stability of the native complex, *O. australiensis* RCA is co-limited by both RCA-Rubisco and self-aggregation stability at a much higher temperature than *O. sativa*.

[054] **Figure 22.** Activation of wheat and rice Rubisco by RCA from rice. The specific activity of Rubisco from *O. sativa* ($Rubisco_{Os}$), Rubisco from *O. australiensis* ($Rubisco_{Oa}$) and Rubisco from wheat ($Rubisco_{wheat}$) (Panel A) and RCA interaction with $Rubisco_{wheat}$ (Panels B to C). Panel A shows the specific activity of fully carbamylated wheat, *O. sativa* (Os) or *O. australiensis* (Oa) Rubisco (closed symbols) and the spontaneous activity of inhibited Rubisco without addition of RCA (open symbols). Panel B shows the temperature-dependent activation of wheat Rubisco by the beta isoform of RCA extracted from wheat,

Os or Oa leaves. The RCA was partially purified from leaf tissue using ammonium sulphate precipitation followed by an extra anion-exchange purification step as described in Example 19. Briefly, desalted RCA leaf extract was passed through a strong anion-exchange column (Q-Sepharose) followed by elution with 0.2 M KCl, which allowed for separation and collection of the beta isoform of RCA. 0.4 mg mL^{-1} of RCA extract was used per assay. Panel C is the concentration-dependent activation of wheat Rubisco by recombinant RCA_{Os} (blue circles) and RCA_{Oa} (red squares). The solid and dashed curves are the RCA_{Os} and RCA_{Oa} recombinant RCA concentration-dependent activation of $Rubisco_{Os}$, respectively. For Panel A and B the means and SE of three experimental replicates are given, while in Panel C the individual observations are plotted.

[055] **Figure 23.** Shows temperature and light settings of the growth chamber for the experiment with transgenic rice lines.

[056] **Figure 24.** Shows the net photosynthetic rates (A_n) (**A**), the maximum carboxylation rates of rubisco (V_{cmax}) (**B**) and the induction kinetics for rubisco after dark-light transition (A_n/min) (**C**) of corresponding transgenic and azygous rice lines (rcaIIOa). Boxplots with means and standard deviations are shown for all parameters (n ≥ 6). Asterisks indicate significant difference between homozygous rcaIIOa single copy line and its corresponding null ($p \leq 0.05$).

[057] **Figure 25.** Shows the dry mass increase during the first 35 days of seedling growth for *O. sativa* and *O. australiensis* at three day temperatures (25°C, 35°C and 45°C). Night temperatures were set at 21°C. Dry biomass was then used to calculate relative growth rates (RGR) the 7- 35 day interval. RGR for *O. sativa* from 7 - 28 days at 45°C is represented by the single dot, while the arrest of growth after 28 days suppressed RGR. Bars indicate standard errors of the mean, while box plots show means with ranges and Tukey numbers to indicate significant differences.

[058] **Figure 26.** Shows net assimilation rate (NAR) and specific leaf area (SLA) plotted against relative growth rate on a biomass basis (RGR) using observations from all temperatures (25, 35 and 45°C) and two species (*O. sativa* and *O. australiensis*). The data show a significant correlation between NAR and RGR ($r^2 = 0.76$; *O. sativa*; 0.95 *O. australiensis*), indicating assimilation (vis-à-vis leaf morphology) to be the driving variable for growth of these young rice seedlings. Data points are shown with standard error of the mean.

[059] **Figure 27.** DNA sequence alignment of Rubisco activase for *O. sativa* ssp. japonica cv. Nipponbare (NCBI accession number; AB 110180) with *O. sativa* cv. Amaroo, *O. meridionalis* cy, *O. meridionalis* kr and *O. australiensis*. Shaded residues represent nucleotide differences. Positions 258, 666 and 774 are differences between *O. sativa* and

O. meridionalis. Sequence differences between *O. sativa* and *both O. meridionalis* and *O. australiensis* are also seen at positions 669, 1017 and 1065. Remaining shaded residues in the *O. australiensis* sequence represent differences between *O. sativa* and *O. australiensis*.

[060] **Figure 28.** Amino acid alignment between the short form (NCBI accession number; BAA97584.1) and long form (NCBI accession number; BAC78572.1) of Rubisco activase from *O. sativa* ssp. japonica cv. Nipponbare and the long form of *O. sativa* cv. Amaroo, *O. meridionalis* cy, *O. meridionalis* kr and *O. australiensis*. Note that the only difference between the two forms is the extra 40 residues at the carboxyl terminal of the long form of activase. Shading shows residues that differ between the protein sequences.

[061] **Figure 29.** Amino acid alignment of *O. sativa* cv. Amaroo and *O. australiensis* with the warm-adapted species *Gossypium hirsutum* (cotton) and *Larrea tridentata* (creosote bush) and the cold-adapted species *Spinacia oleracea* (spinach) *and Deschampsia antarctica* (Antarctic hairgrass). Shading shows residues that *O. australiensis* has in common with the warm-adapted species that are not present in *O. sativa* or the two cold-adapted species.

[062] **Figure 30.** Tiller numbers measured for each genotype across all 14 blocks after seven weeks of treatment at 45°C daily. Boxplots are given, as described in Figure 24.

[063] **Figure 31.** Results of tillers per plant for eight lines counted in six-week-old plants that had been exposed to one week at 45^{O}C day temperatures as described in Figure 23. Lines 1-8 are listed in the order, 474, 472, 478, 476, 473, 475, 480 (control) and 479, respectively.

Mode(s) for Carrying Out the Invention

[064] The present invention relates to RCA (Rubisco activase) and functionally equivalent polypeptides that confer thermostability to a complex comprising RCA (or a functionally equivalent polypeptide) and Rubisco.

Rubisco Activase (RCA) Polypeptides and Nucleic Acids

[065] In some embodiments, the complexes comprise *O. australiensis* RCA or an RCA comprising at least one of SEQ ID NOs: 2-37, 39, 40 and 50 to 52 in Table 1, Table 2 and Table 6.

Table 1. Sequence identification

SEQ ID NO	Sequence	Relative position*	change in Oa occurs at the following corresponding position in the Os seq	High heat stress[a]	High heat stress[b]
1	See Table 3	NA			
2	ELDE**X**$_1$KQTD	E^{50}LDE**X**$_1$KQTD	54	+	+
3	ELDE**D**KQTD	E^{50}LDE**D**KQTD	54	+	+
4	TDQD**K**WKGL	T^{57}DQD**K**WKGL	61	+	
5	E**X**$_1$KQTDQD**K**W	E^{53}**X**$_1$KQTDQD**K**W	54, 61	+	
6	E**D**KQTDQD**K**W	E^{53}**D**KQTDQD**K**W	54, 61	+	
7	RGKG**X**$_2$VDSL	R^{78}GKG**X**$_2$VDSL	82	+	
8	RGKG**L**VDSL	R^{78}GKG**L**VDSL	82	+	
9	FQAP**X**$_3$GDGT	F^{87}QAP**X**$_3$GDGT	91	+	
10	FQAP**M**GDGT	F^{87}QAP**M**GDGT	91	+	
11	G**X**$_2$VDSLFQAP**X**$_3$	G^{81}**X**$_2$VDSLFQAP**X**$_3$	82, 91	+	
12	G**L**VDSLFQAP**M**	G^{81}**L**VDSLFQAP**M**	82, 91	+	
13	EYLSQGL**K**	E^{104}YLSQGL**K**	111	+	
14	YLSQGL**KX**$_1$	Y^{105}LSQGL**KX**$_1$	111,112	+	
15	YLSQGL**KM**	Y^{105}LSQGL**KM**	111,112	+	
16	LSQGL**KX**$_1$**X**$_2$	L^{106}SQGL**KX**$_1$**X**$_2$	111-113	+	
17	LSQGL**KML**	L^{106}SQGL**KML**	111-113	+	
18	**KX**$_1$**X**$_2$DNTMGGFY	**K**^{111}**X**$_1$**X**$_2$DNTMGGFY	111-113	+	
19	**KML**DNTMGGFY	**K**^{111}**ML**DNTMGGFY	111-113	+	
20	**X**$_1$**X**$_2$DNTMGGFYI	**X**$_1$^{112}**X**$_2$DNTMGGFYI	112-113	+	+
21	**ML**DNTMGGFYI	**M**^{112}**X**$_2$DNTMGGFYI	112-113	+	+
22	**X**$_2$DNTMGGFYIA	**X**$_2$^{113}DNTMGGFYIA	113	+	+

SEQ ID NO	Sequence	Relative position*	change in Oa occurs at the following corresponding position in the Os seq	High heat stress[a]	High heat stress[b]
23	**L**DNTMGGFYIA	**L**^{113}DNTMGGFYIA	113	+	+
24	SQGL**KX$_1$X$_2$**DNTM	S^{107}QGL**KX$_1$X$_2$**DNTM	111-113	+	+
25	SQGL**KML**DNTM	S^{107}QGL**KML**DNTM	111-113	+	+
26	KNFM**X$_4$**LPNI	K^{138}NFM**X$_4$**LPNI	142	+	
27	KNFM**A**LPNI	K^{138}NFM**A**LPNI	142	+	
28	ELVF**S**KMGI	E^{167}LVF**S**KMGI	171	+	
29	NIGK**K**LVNS	N^{359}IGK**K**LVNS	363	+	
30	KMTI**D**KLME	K^{378}MTI**D**KLME	382	+	+
31	MEYG**H**MLY	M^{385}EYG**H**MLY	389	+	
32	MLV**R**EQEN	M^{390}LV**R**EQEN	393	+	
33	**D**KLMEYG**H**MLV**R**	**D**^{382}KLMEYG**H**MLV**R**	382, 389,393	+	
34	NVKRVQLA**D**	N^{397}VKRVQLA**D**	405	+	
35	**K**YLSEAALG	**K**^{406}YLSEAALG	406	+	+
36	VQLA**DK**YLSE	V^{401}QLA**DK**YLSE	405,406	+	+
37	GAQQ**G**GNLP	G^{430}AQQ**G**GNLP	434	+	
38	See Table 2	NA			
39	See Table 2	NA			
40	See Table 2	NA			
41	See Table 3	NA			
42	See Table 3	NA			
43	See Table 2	NA			
44	See Table 3	NA			
45	SFYGAAPSS	S^{425}FYGAAPSS	429		

SEQ ID NO	Sequence	Relative position*	change in Oa occurs at the following corresponding position in the Os seq	High heat stress[a]	High heat stress[b]
46	See Table 5	NA			
47	See Table 5	NA			
48	See Table 5	NA			
49	See Table 5	NA			
50	See Table 6	NA			
51	See Table 6	NA			
52	See Table 6	NA			
53	See Table 7	NA			

X_1 = charged (K, R, N, M, Q, H, D, E)

X_2 = aliphatic (L, I, V)

X_3 = positive charged (K, R, N, M, Q, H)

X_4 = small (A, G)

a = residue found in relevant position in *O. australiensis* Rubisco activase

b = residue found in analogous position in Rubisco activase of warm-adapted species *Gossypium hirsutum* (cotton) and *Larrea tridentate* (creosote bush).

* = Position relative to SEQ ID No: 38 (BAC78572.1)

Table 2. Rubisco Activase RCA (RCA) polypeptide Sequences

SEQ ID NO:	Sequence
38	MAAAFSSTVGAPASTPTNFLGKKLKKQVTSAVNYHGKSSNINRFKVMAK ELDEGKQTDQDRWKGLAYDISDDQQDITRGKGFVDSLFQAPTGDGTHEA VLSSYEYLSQGLRTYDFDNTMGGFYIAPAFMDKLVVHISKNFMTLPNIK VPLILGIWGGKGQGKSFQCELVFAKMGINPIMMSAGELESGNAGEPAKL IRQRYREAADIIKKGKMCCLFINDLDAGAGRMGGTTQYTVNNQMVNATL MNIADNPTNVQLPGMYNKEDNPRVPIIVTGNDFSTLYAPLIRDGRMEKF YWAPTRDDRVGVCKGIFRTDNVPDEDIVKIVDSFPGQSIDFFGALRARV YDDEVRKWVSDTGVENIGKRLVNSREGPPEFEQPKMTIEKLMEYGYMLV KEQENVKRVQLAEQYLSEAALGDANSDAMKTGSFYGSAPSS

SEQ ID NO:	Sequence
39	MAAAFSSTVGAPASTPTNFLGKKLKKQVTSAVNYHGKSSNINRFKVMAK ELDEDKQTDQDKWKGLAYDISDDQQDITRGKGLVDSLFQAPMGDGTHEA VLSSYEYLSQGLKMLDNTMGGFYIAPAFMDKLVVHISKNFMALPNIKVP LILGIWGGKGQGKSFQCELVFSKMGINPIMMSAGELESGNAGEPAKLIR QRYREAADIIKKGKMCCLFINDLDAGAGRMGGTTQYTVNNQMVNATLMN IADNPTNVQLPGMYNKEDNPRVPIIVTGNDFSTLYAPLIRDGRMEKFYW APTRDDRVGVCKGIFRTDNVPDEDIVKIVDSFPGQSIDFFGALRARVYD DEVRKWVSDTGVENIGKKLVNSREGPPEFEQPKMTIDKLMEYGHMLVRE QENVKRVQLADKYLSEAALGDANSDAMKTGSFYGQGAQQGGNLPVPEGC TDPVAKNFDPTARSDDGSCLYTF
40	MAAAFSSTVGAPASTPTNFLGKKLKKQVTSAVNYHGKSSNINRFKVMAK ELDEDKQTDQDKWKGLAYDISDDQQDITRGKGLVDSLFQAPMGDGTHEA VLSSYEYLSQGLKMLDNTMGGFYIAPAFMDKLVVHISKNFMALPNIKVP LILGIWGGKGQGKSFQCELVFSKMGINPIMMSAGELESGNAGEPAKLIR QRYREAADIIKKGKMCCLFINDLDAGAGRMGGTTQYTVNNQMVNATLMN IADNPTNVQLPGMYNKEDNPRVPIIVTGNDFSTLYAPLIRDGRMEKFYW APTRDDRVGVCKGIFRTDNVPDEDIVKIVDSFPGQSIDFFGALRARVYD DEVRKWVSDTGVENIGKKLVNSREGPPEFEQPKMTIDKLMEYGHMLVRE QENVKRVQLADKYLSEAALGDANSDAMKTGSFYGAAPSS
43	MAAAFSSTVGAPASTPTNFLGKKLKKQVTSAVNYHGKSSNINRFKVMAK ELDEGKQTDQDRWKGLAYDISDDQQDITRGKGFVDSLFQAPTGDGTHEA VLSSYEYLSQGLRTYDFDNTMGGFYIAPAFMDKLVVHISKNFMTLPNIK VPLILGIWGGKGQGKSFQCELVFAKMGINPIMMSAGELESGNAGEPAKL IRQRYREAADIIKKGKMCCLFINDLDAGAGRMGGTTQYTVNNQMVNATL MNIADNPTNVQLPGMYNKEDNPRVPIIVTGNDFSTLYAPLIRDGRMEKF YWAPTRDDRVGVCKGIFRTDNVPDEDIVKIVDSFPGQSIDFFGALRARV YDDEVRKWVSDTGVENIGKRLVNSREGPPEFEQPKMTIEKLMEYGYMLV KEQENVKRVQLAEQYLSEAALGDANSDAMKTGSFYGQGAQQAGNLPVPE GCTDPVAKNFDPTARSDDGSCLYTF

Table 3. Rubisco Activase (RCA) nucleotide sequences

SEQ ID NO:	Sequence
1	ATGGCTGCTGCCTTCTCCTCCACCGTTGGAGCTCCGGCGTCCACTCCGA CCAACTTCCTGGGGAAGAAGCTGAAAAAGCAGGTGACATCGGCGGTGAA CTACCATGGCAAGAGCTCCAACATCAACAGGTTCAAGGTGATGGCCAAG GAGCTGGACGAGGGCAAGCAGACCGACCAGGACAGGTGGAAGGGTCTCG CCTACGACATCTCCGATGACCAGCAGGACATCACCAGGGGGAAGGGTTT CGTCGACTCCCTTTTCCAGGCTCCCACGGGTGATGGCACCCACGAGGCC GTCCTCAGCTCCTACGAGTACCTCAGCCAGGGTCTCAGAACGTACGACT TCGACAACACCATGGGAGGCTTCTACATCGCCCCTGCTTTCATGGACAA GCTCGTCGTCCACATCTCCAAGAACTTCATGACCCTCCCCAACATCAAG

SEQ ID NO:	Sequence
	GTCCCACTCATCCTGGGTATCTGGGGAGGCAAGGGTCAGGGAAAATCCT TCCAGTGTGAGCTCGTCTTCGCCAAGATGGGGATCAACCCCATCATGAT GAGCGCCGGAGAGCTGGAGAGCGGCAACGCCGGAGAGCCGGCGAAGCTG ATCAGGCAGCGGTACCGTGAGGCGGCAGACATCATCAAGAAGGGGAAGA TGTGCTGCCTCTTCATCAACGATCTGGACGCGGGTGCAGGTCGCATGGG AGGCACCACCCAGTACACGGTGAACAACCAGATGGTGAACGCCACCCTG ATGAACATCGCCGACAACCCAACCAACGTGCAGCTCCCCGGGATGTACA ACAAGGAGGACAACCCCCGTGTCCCCATCATCGTCACCGGCAACGACTT CTCCACGCTGTACGCGCCGCTCATCCGTGACGGGCGTATGGAGAAGTTC TACTGGGCTCCCACCCGCGACGACCGTGTCGGCGTCTGCAAGGGTATCT TCCGCACCGACAACGTCCCCGACGAGGACATCGTCAAGATCGTCGACAG CTTCCCAGGCCAATCCATCGATTTCTTCGGCGCTCTTCGTGCCCGTGTT TACGACGACGAGGTGCGCAAGTGGGTGTCGGACACGGGTGTGGAGAACA TTGGCAAGAGGCTGGTGAACTCGAGGGAGGGCCCACCGGAGTTCGAGCA GCCCAAGATGACGATCGAAAAGCTCATGGAGTACGGATACATGCTTGTG AAGGAGCAGGAGAACGTCAAGCGTGTGCAGCTGGCTGAGCAGTACTTGA GCGAGGCTGCTCTTGGTGACGCTAACTCCGACGCCATGAAGACTGGTTC CTTCTACGGTTCTGCGCCATCCAGCTGA
41	ATGGCTGCTGCCTTCTCCTCCACCGTTGGAGCTCCGGCCTCCACTCCGA CCAACTTCCTGGGGAAGAAGCTGAAGAAGCAGGTGACATCGGCCGTGAA CTACCATGGCAAGAGCTCCAACATCAACAGGTTCAAAGTGATGGCCAAG GAGCTCGACGAGGACAAGCAGACCGACCAGGACAAGTGGAAGGGTCTCG CCTACGACATCTCCGATGACCAGCAGGACATCACCAGGGGGAAGGGTCT CGTCGACTCCCTCTTCCAGGCTCCCATGGGTGATGGCACCCACGAGGCC GTCCTCAGCTCCTACGAGTACCTCAGCCAGGGTCTCAAAATGTTGGACA ACACCATGGGAGGCTTCTACATCGCCCCGGCTTTCATGGACAAGCTCGT CGTCCACATCTCCAAGAACTTCATGGCCCTCCCCAACATCAAGGTCCCA CTCATCCTGGGTATCTGGGGAGGCAAGGGTCAGGGAAAATCCTTCCAGT GTGAGCTCGTCTTCTCCAAGATGGGGATCAACCCCATCATGATGAGTGC CGGAGAGCTGGAGAGCGGCAACGCCGGAGAGCCCGCGAAGCTCATCAGG CAGCGGTATCGTGAGGCGGCGGACATCATCAAGAAGGGGAAGATGTGCT GCCTCTTCATCAACGATCTCGACGCCGGAGCAGGTCGCATGGGCGGCAC CACCCAGTACACGGTGAACAACCAGATGGTGAACGCCACCCTGATGAAC ATCGCCGACAACCCAACCAACGTGCAGCTGCCGGGCATGTACAACAAGG AGGACAACCCCCGTGTCCCCATCATCGTCACCGGTAACGACTTCTCGAC GCTGTACGCGCCGCTCATCCGTGACGGGCGTATGGAGAAGTTCTACTGG GCTCCCACCCGCGATGACCGTGTCGGCGTCTGCAAGGGTATCTTCCGCA CCGACAACGTCCCCGACGAGGACATCGTCAAAATCGTGGACAGCTTCCC AGGCCAATCCATCGATTTCTTCGGTGCTCTGCGTGCCCGTGTTTACGAC GACGAGGTGCGCAAGTGGGTGTCGGACACCGGTGTGGAGAACATTGGCA AGAAGCTGGTGAACTCGAGGGAGGGCCCACCGGAGTTCGAGCAGCCCAA GATGACGATCGACAAGCTGATGGAGTACGGACACATGCTTGTGAGGGAG CAGGAGAACGTCAAGCGTGTGCAGCTGGCTGACAAGTACTTGAGCGAGG CTGCTCTTGGTGACGCTAACTCCGACGCCATGAAGACTGGTTCCTTCTA CGGTGCTGCGCCATCCAGCTGA

SEQ ID NO:	Sequence
42	ATGGCTGCTGCCTTCTCCTCCACCGTTGGAGCTCCGGCCTCCACTCCGA CCAACTTCCTGGGGAAGAAGCTGAAGAAGCAGGTGACATCGGCCGTGAA CTACCATGGCAAGAGCTCCAACATCAACAGGTTCAAAGTGATGGCCAAG GAGCTCGACGAGGACAAGCAGACCGACCAGGACAAGTGGAAGGGTCTCG CCTACGACATCTCCGATGACCAGCAGGACATCACCAGGGGGAAGGGTCT CGTCGACTCCCTCTTCCAGGCTCCCATGGGTGATGGCACCCACGAGGCC GTCCTCAGCTCCTACGAGTACCTCAGCCAGGGTCTCAAAATGTTGGACA ACACCATGGGAGGCTTCTACATCGCCCCGGCTTTCATGGACAAGCTCGT CGTCCACATCTCCAAGAACTTCATGGCCCTCCCCAACATCAAGGTCCCA CTCATCCTGGGTATCTGGGGAGGCAAGGGTCAGGGAAAATCCTTCCAGT GTGAGCTCGTCTTCTCCAAGATGGGGATCAACCCCATCATGATGAGTGC CGGAGAGCTGGAGAGCGGCAACGCCGGAGAGCCCGCGAAGCTCATCAGG CAGCGGTATCGTGAGGCGGCGGACATCATCAAGAAGGGGAAGATGTGCT GCCTCTTCATCAACGATCTCGACGCCGGAGCAGGTCGCATGGGCGGCAC CACCCAGTACACGGTGAACAACCAGATGGTGAACGCCACCCTGATGAAC ATCGCCGACAACCCAACCAACGTGCAGCTGCCGGGCATGTACAACAAGG AGGACAACCCCCGTGTCCCCATCATCGTCACCGGTAACGACTTCTCGAC GCTGTACGCGCCGCTCATCCGTGACGGGCGTATGGAGAAGTTCTACTGG GCTCCCACCCGCGATGACCGTGTCGGCGTCTGCAAGGGTATCTTCCGCA CCGACAACGTCCCCGACGAGGACATCGTCAAAATCGTGGACAGCTTCCC AGGCCAATCCATCGATTTCTTCGGTGCTCTGCGTGCCCGTGTTTACGAC GACGAGGTGCGCAAGTGGGTGTCGGACACCGGTGTGGAGAACATTGGCA AGAAGCTGGTGAACTCGAGGGAGGGCCCACCGGAGTTCGAGCAGCCCAA GATGACGATCGACAAGCTGATGGAGTACGGACACATGCTTGTGAGGGAG CAGGAGAACGTCAAGCGTGTGCAGCTGGCTGACAAGTACTTGAGCGAGG CTGCTCTTGGTGACGCTAACTCCGACGCCATGAAGACTGGTTCCTTCTA CGGGCAAGGAGCACAGCAAGGAGGTAACCTGCCTGTGCCGGAAGGTTGC ACCGACCCTGTTGCCAAGAACTTCGACCCAACGGCGAGGAGCGACGACG GCAGCTGCCTTTACACCTTTTAA
44	ATGGCTGCTGCCTTCTCCTCCACCGTTGGAGCTCCGGCGTCCACTCCGA CCAACTTCCTGGGGAAGAAGCTGAAGAAGCAGGTGACATCGGCGGTGAA CTACCATGGCAAGAGCTCCAACATCAACAGGTTCAAGGTGATGGCCAAG GAGCTGGACGAGGGCAAGCAGACCGACCAGGACAGGTGGAAGGGTCTCG CCTACGACATCTCCGATGACCAGCAGGACATCACCAGGGGGAAGGGTTT CGTCGACTCCCTTTTCCAGGCTCCCACGGGTGATGGCACCCACGAGGCC GTCCTCAGCTCCTACGAGTACCTCAGCCAGGGTCTCAGAACGTACGACT TCGACAACACCATGGGAGGCTTCTACATCGCCCCTGCTTTCATGGACAA GCTCGTCGTCCACATCTCCAAGAACTTCATGACCCTCCCCAACATCAAG GTCCCACTCATCCTGGGTATCTGGGGAGGCAAGGGTCAGGGAAAATCCT TCCAGTGTGAGCTCGTCTTCGCCAAGATGGGGATCAACCCCATCATGAT GAGCGCCGGAGAGCTGGAGAGCGGCAACGCCGGAGAGCCGGCGAAGCTG ATCAGGCAGCGGTACCGTGAGGCGGCAGACATCATCAAGAAGGGGAAGA TGTGCTGCCTCTTCATCAACGATCTGGACGCGGGTGCAGGTCGCATGGG AGGCACCACCCAGTACACGGTGAACAACCAGATGGTGAACGCCACCCTG ATGAACATCGCCGACAACCCAACCAACGTGCAGCTCCCCGGGATGTACA ACAAGGAGGACAACCCCCGTGTCCCCATCATCGTCACCGGCAACGACTT CTCCACGCTGTACGCGCCGCTCATCCGTGACGGGCGTATGGAGAAGTTC

SEQ ID NO:	Sequence
	TACTGGGCTCCCACCCGCGACGACCGTGTCGGCGTCTGCAAGGGTATCT TCCGCACCGACAACGTCCCCGACGAGGACATCGTCAAGATCGTCGACAG CTTCCCAGGCCAATCCATCGATTTCTTCGGCGCTCTTCGTGCCCGTGTT TACGACGACGAGGTGCGCAAGTGGGTGTCGGACACGGGTGTGGAGAACA TTGGCAAGAGGCTGGTGAACTCGAGGGAGGGCCCACCGGAGTTCGAGCA GCCCAAGATGACGATCGAAAAGCTCATGGAGTACGGATACATGCTTGTG AAGGAGCAGGAGAACGTCAAGCGTGTGCAGCTGGCTGAGCAGTACTTGA GCGAGGCTGCTCTTGGTGACGCTAACTCCGACGCCATGAAGACTGGTTC CTTCTACGGGCAAGGAGCACAGCAAGCAGGTAACCTGCCTGTGCCGGAA GGTTGCACCGACCCTGTTGCCAAGAACTTCGACCCAACGGCGAGGAGCG ACGACGGCAGCTGCCTTTACACCTTTTAA

Table 4. Sequence information

SEQ ID NO:	Information
1	Nucleotide sequence of short form of Rubisco activase from *O. sativa* ssp. japonica cv. Nipponbare
38	Amino acid sequence Rubisco activase from *O. sativa* ssp. japonica cv. Nipponbare (>gi |8918361| dbj | BAA97584 .1| ribulose-bisphosphate carboxylase activase small isoform precursor protein [*Oryza sativa* japonica subspecies])
39	Amino acid sequence of long form of Rubisco activase from *O. australiensis*
40	Amino acid sequence of short form of Rubisco activase from *O. australiensis*
41	Nucleotide sequence of short form of Rubisco activase nucleic acid from *O. australiensis*
42	Nucleotide sequence of long form of Rubisco activase nucleic acid from *O. australiensis*
43	Amino acid sequence of Rubisco activase from *O. sativa* ssp. japonica cv. Nipponbare (>gi |32352158| dbj | BAC78572.1) Rubisco activase long isoform precursor protein [*Oryza sativa* japonica subspecies])
44	Nucleotide sequence of long form of Rubisco activase from *O. sativa* ssp. japonica cv. Nipponbare
50	Nucleotide sequence of long form of Rubisco activase nucleic acid from *O. australiensis* (adapted to rice codon usage), used for vector pTNVS299
51	Nucleotide sequence of short form of Rubisco activase nucleic acid from *O. australiensis* (adapted to rice codon usage) with a 5AA truncation at the end of the gene, used for vector pTKAW39
52	Nucleotide sequence of short form of Rubisco activase nucleic acid from *O. australiensis* (adapted to rice codon usage), used for vector pTKAW40
53	Nucleotide sequence of the promoter of Rubisco activase from *O. meridionalis*, used for vectors pTNVS299, pTKAW39, pTKAW40

54	Nucleotide sequence of Rubisco activase from *O. meridionalis*
55	Amino acid sequence of *O. meridionalis* alpha protein
56	Amino acid sequence of *O. meridionalis* beta protein
57	Amino acid sequence of *Descampsia antarctica* protein
58	Amino acid sequence of *Gossypium hirsutum* protein
59	Amino acid sequence of *Larrea tridentata* protein
60	Amino acid sequence of *Spinacia oleracea* protein

Table 5. RCA genes adapted to *E. coli* usage

SEQ ID NO:	**Sequence**
46	ATGGCTAAAGAACTGGACGAAGGCAAACAGACTGATCAGGACCGCTGGA AAGGTCTGGCCTACGATATTTCCGATGACCAGCAGGATATCACCCGCGG TAAAGGCTTCGTAGATTCTCTGTTCCAGGCGCCAACTGGTGATGGTACT CATGAAGCGGTTCTGAGCTCCTACGAATATCTGAGCCAGGGTCTGCGTA CCTATGACTTCGACAACACTATGGGCGGTTTTTACATCGCTCCGGCTTT CATGGACAAACTGGTCGTCCACATCTCCAAAAACTTCATGACCCTGCCG AACATCAAAGTGCCACTGATTCTCGGCATCTGGGGTGGCAAAGGTCAGG GCAAATCCTTCCAGTGTGAACTGGTCTTCGCGAAAATGGGCATTAACCC GATCATGATGAGCGCAGGTGAACTGGAATCTGGTAACGCCGGTGAACCG GCGAAACTGATTCGTCAGCGTTACCGTGAAGCGGCCGACATCATCAAAA AAGGCAAAATGTGCTGCCTGTTCATCAACGATCTGGATGCTGGTGCAGG TCGTATGGGTGGCACTACCCAGTATACCGTGAACAACCAGATGGTTAAC GCCACCCTGATGAACATTGCCGATAACCCGACTAACGTACAGCTGCCGG GCATGTATAACAAAGAAGACAACCCGCGCGTTCCGATCATTGTTACCGG CAACGACTTCTCTACGCTGTATGCTCCGCTGATTCGTGATGGCCGTATG GAGAAATTCTACTGGGCACCAACCCGTGACGACCGTGTTGGTGTATGCA AAGGTATCTTCCGCACCGATAACGTTCCGGACGAAGACATCGTCAAAAT CGTGGACAGCTTTCCGGGCCAGTCTATCGATTTCTTTGGCGCTCTGCGT GCTCGTGTTTACGACGACGAAGTTCGCAAATGGGTGTCTGATACGGGTG TGGAAAACATCGGCAAACGTCTGGTTAACTCTCGTGAAGGTCCACCGGA GTTCGAACAGCCGAAAATGACGATCGAGAAACTGATGGAGTACGGCTAC ATGCTGGTGAAAGAGCAGGAGAACGTGAAACGTGTACAGCTGGCAGAAC AGTACCTGAGCGAAGCTGCACTGGGTGATGCGAACTCTGACGCGATGAA AACTGGCTCCTTTTATGGTCAGGGTGCACAGCAGGCAGGTAACCTCCCA GTACCAGAAGGTTGTACCGACCCAGTTGCGAAAAACTTTGACCCAACCG CTCGCTCTGATGACGGTTCTTGCCTGTACACCTTCTAA
47	ATGGCCAAAGAACTGGACGAAGACAAACAGACCGACCAGGACAAATGGA AAGGTCTGGCGTACGACATCTCTGATGACCAGCAGGATATCACCCGTGG CAAAGGCCTGGTAGATTCTCTGTTCCAGGCTCCGATGGGTGATGGTACT CATGAGGCGGTTCTGTCTTCCTATGAGTACCTGAGCCAGGGCCTGAAAA TGCTGGATAACACTATGGGTGGCTTCTACATTGCACCGGCCTTTATGGA CAAACTGGTCGTCCACATCTCCAAAAACTTCATGGCCCTGCCGAACATC AAAGTCCCGCTGATTCTGGGTATTTGGGGTGGCAAAGGTCAGGGCAAAT

SEQ ID NO:	Sequence
	CCTTCCAGTGCGAACTGGTCTTCAGCAAAATGGGCATCAACCCGATCAT GATGAGCGCTGGTGAACTGGAATCTGGTAACGCTGGCGAACCGGCTAAA CTGATTCGTCAGCGTTACCGCGAAGCGGCCGACATCATCAAAAAAGGCA AAATGTGCTGCCTGTTCATCAACGATCTGGATGCAGGTGCAGGTCGTAT GGGTGGCACTACCCAGTATACCGTGAACAACCAGATGGTGAACGCGACC CTGATGAACATCGCGGATAACCCAACCAACGTTCAGCTGCCGGGCATGT ACAACAAAGAAGACAACCCGCGTGTCCCGATTATCGTTACCGGCAACGA CTTCTCCACTCTCTATGCTCCGCTGATCCGTGACGGCCGTATGGAAAAA TTCTACTGGGCACCGACTCGCGACGATCGTGTAGGCGTGTGTAAAGGTA TCTTCCGTACCGACAACGTCCCAGACGAGGACATTGTGAAAATCGTGGA CAGCTTTCCGGGCCAGTCTATTGATTTCTTTGGCGCTCTGCGTGCACGT GTATACGACGATGAAGTCCGCAAATGGGTTAGCGACACGGGTGTTGAGA ACATCGGCAAAAAACTGGTGAACTCCCGTGAAGGTCCACCGGAATTTGA GCAGCCGAAAATGACGATCGACAAACTGATGGAGTACGGTCACATGCTG GTACGCGAACAGGAAAACGTGAAACGTGTGCAGCTGGCGGATAAATACC TGAGCGAAGCAGCTCTGGGTGATGCTAACTCCGATGCGATGAAAACCGG CTCTTTCTATGGTCAGGGTGCACAGCAGGGCGGTAACCTGCCAGTACCG GAAGGTTGTACTGATCCGGTTGCGAAAAACTTCGATCCAACGGCTCGCT CTGATGACGGTTCTTGCCTCTATACCTTTGCGGGTCGCTAA
48	ATGGCTAAAGAACTGGACGAAGGCAAACAGACTGATCAGGACCGCTGGA AAGGTCTGGCCTACGATATTTCCGATGACCAGCAGGATATCACCCGCGG TAAAGGCTTCGTAGATTCTCTGTTCCAGGCGCCAACTGGTGATGGTACT CATGAAGCGGTTCTGAGCTCCTACGAATATCTGAGCCAGGGTCTGCGTA CCTATGACTTCGACAACACTATGGGCGGTTTTTACATCGCTCCGGCTTT CATGGACAAACTGGTCGTCCACATCTCCAAAAACTTCATGACCCTGCCG AACATCAAAGTGCCACTGATTCTCGGCATCTGGGGTGGCAAAGGTCAGG GCAAATCCTTCCAGTGTGAACTGGTCTTCGCGAAAATGGGCATTAACCC GATCATGATGAGCGCAGGTGAACTGGAATCTGGTAACGCCGGTGAACCG GCGAAACTGATTCGTCAGCGTTACCGTGAAGCGGCCGACATCATCAAAA AAGGCAAAATGTGCTGCCTGTTCATCAACGATCTGGATGCTGGTGCAGG TCGTATGGGTGGCACTACCCAGTATACCGTGAACAACCAGATGGTTAAC GCCACCCTGATGAACATTGCCGATAACCCGACTAACGTACAGCTGCCGG GCATGTATAACAAAGAAGACAACCCGCGCGTTCCGATCATTGTTACCGG CAACGACTTCTCTACGCTGTATGCTCCGCTGATTCGTGATGGCCGTATG GAGAAATTCTACTGGGCACCAACCCGTGACGACCGTGTTGGTGTATGCA AAGGTATCTTCCGCACCGATAACGTTCCGGACGAAGACATCGTCAAAAT CGTGGACAGCTTTCCGGGCCAGTCTATCGATTTCTTTGGCGCTCTGCGT GCTCGTGTTTACGACGACGAAGTTCGCAAATGGGTGTCTGATACGGGTG TGGAAAACATCGGCAAACGTCTGGTTAACTCTCGTGAAGGTCCACCGGA GTTCGAACAGCCGAAAATGACGATCGAGAAACTGATGGAGTACGGCTAC ATGCTGGTGAAAGAGCAGGAGAACGTGAAACGTGTACAGCTGGCAGAAC AGTACCTGAGCGAAGCTGCACTGGGTGATGCGAACTCTGACGCGATGAA AACTGGCTCCTTTTATGGTTCCGCGCCGTCTTCCTAA

SEQ ID NO:	Sequence
49	ATGGCCAAAGAACTGGACGAAGACAAACAGACCGACCAGGACAAATGGA AAGGTCTGGCGTACGACATCTCTGATGACCAGCAGGATATCACCCGTGG CAAAGGCCTGGTAGATTCTCTGTTCCAGGCTCCGATGGGTGATGGTACT CATGAGGCGGTTCTGTCTTCCTATGAGTACCTGAGCCAGGGCCTGAAAA TGCTGGATAACACTATGGGTGGCTTCTACATTGCACCGGCCTTTATGGA CAAACTGGTCGTCCACATCTCCAAAAACTTCATGGCCCTGCCGAACATC AAAGTCCCGCTGATTCTGGGTATTTGGGGTGGCAAAGGTCAGGGCAAAT CCTTCCAGTGCGAACTGGTCTTCAGCAAAATGGGCATCAACCCGATCAT GATGAGCGCTGGTGAACTGGAATCTGGTAACGCTGGCGAACCGGCTAAA CTGATTCGTCAGCGTTACCGCGAAGCGGCCGACATCATCAAAAAAGGCA AAATGTGCTGCCTGTTCATCAACGATCTGGATGCAGGTGCAGGTCGTAT GGGTGGCACTACCCAGTATACCGTGAACAACCAGATGGTGAACGCGACC CTGATGAACATCGCGGATAACCCAACCAACGTTCAGCTGCCGGGCATGT ACAACAAAGAAGACAACCCGCGTGTCCCGATTATCGTTACCGGCAACGA CTTCTCCACTCTCTATGCTCCGCTGATCCGTGACGGCCGTATGGAAAAA TTCTACTGGGCACCGACTCGCGACGATCGTGTAGGCGTGTGTAAAGGTA TCTTCCGTACCGACAACGTCCCAGACGAGGACATTGTGAAAATCGTGGA CAGCTTTCCGGGCCAGTCTATTGATTTCTTTGGCGCTCTGCGTGCACGT GTATACGACGATGAAGTCCGCAAATGGGTTAGCGACACGGGTGTTGAGA ACATCGGCAAAAAACTGGTGAACTCCCGTGAAGGTCCACCGGAATTTGA GCAGCCGAAAATGACGATCGACAAACTGATGGAGTACGGTCACATGCTG GTACGCGAACAGGAAAACGTGAAACGTGTGCAGCTGGCGGATAAATACC TGAGCGAAGCAGCTCTGGGTGATGCTAACTCCGATGCGATGAAAACCGG CTCTTTCTATGGTGCAGCGCCGTCTTCCTAA

Table 6. RCA genes adapted to rice codon usage

SEQ ID NO:	Sequence
50	ATGGCTGCTGCCTTCTCCTCGACAGTTGGAGCACCTGCCTCTACACCGA CAAACTTCCTGGGCAAGAAGCTGAAGAAGCAAGTGACATCCGCCGTGAA CTACCACGGCAAGAGCTCCAACATAAACCGCTTCAAAGTCATGGCGAAG GAGCTCGATGAGGACAAGCAGACCGATCAGGACAAGTGGAAGGGACTTG CCTACGACATCTCCGATGACCAGCAGGACATTACGAGGGGCAAAGGTTT GGTCGACTCCCTTTTCCAAGCACCAATGGGAGATGGCACGCACGAAGCA GTCCTTAGCTCCTACGAGTACCTCTCCCAGGGCTTGAAGATGTTGGACA ACACTATGGGGGGCTTCTACATCGCACCTGCTTTCATGGACAAGCTCGT CGTCCACATCTCGAAGAACTTCATGGCGCTCCCGAACATAAAGGTGCCA CTGATTCTCGGGATCTGGGGAGGCAAGGGTCAGGGGAAATCCTTCCAGT GTGAGCTCGTCTTCTCCAAGATGGGGATCAACCCCATCATGATGAGCGC CGGAGAACTGGAAAGCGGAAATGCCGGAGAACCAGCGAAACTGATCCGA CAGAGATACCGAGAGGCGGCTGACATCATCAAGAAGGGGAAGATGTGCT GCCTCTTCATCAACGATCTGGATGCGGGAGCTGGCCGAATGGGAGGGAC TACTCAATACACCGTCAACAACCAGATGGTGAACGCGACCCTGATGAAC ATCGCGGATAATCCCACCAACGTGCAACTCCCTGGGATGTACAACAAGG AGGACAATCCGCGAGTCCCCATCATCGTCACGGGCAATGATTTCTCGAC ACTCTACGCCCCGCTTATCCGAGATGGGAGGATGGAGAAGTTCTACTGG GCACCTACCAGAGACGATCGCGTAGGTGTCTGCAAAGGCATCTTTCGCA

SEQ ID NO:	Sequence
	CTGACAACGTCCCGGATGAGGACATCGTCAAGATCGTCGACAGCTTCCC TGGCCAATCTATCGACTTTTTCGGCGCTCTACGAGCCCGTGTTTACGAT GACGAAGTGCGGAAATGGGTTTCGGATACGGGCGTGGAGAACATTGGCA AGAAACTGGTGAACTCTAGGGAAGGGCCACCTGAGTTCGAACAGCCGAA GATGACGATCGACAAGCTCATGGAGTACGGCCATATGCTCGTGAGGGAG CAGGAGAACGTTAAGCGTGTTCAACTGGCCGACAAGTACCTAAGCGAGG CAGCTTTGGGTGACGCTAACTCCGACGCCATGAAAACTGGTTCCTTCTA CGGGCAAGGAGCACAGCAAGGCGGAAACTTACCTGTGCCGGAAGGTTGC ACTGACCCCGTTGCGAAGAACTTTGACCCAACGGCGAGATCTGACGATG GGAGCTGCCTTTATACCTTTTAA
51	ATGGCTGCTGCCTTCTCCTCGACAGTTGGAGCACCTGCCTCTACACCGA CAAACTTCCTGGGCAAGAAGCTGAAGAAGCAAGTGACATCCGCCGTGAA CTACCACGGCAAGAGCTCCAACATAAACCGCTTCAAAGTCATGGCGAAG GAGCTCGATGAGGACAAGCAGACCGATCAGGACAAGTGGAAGGGACTTG CCTACGACATCTCCGATGACCAGCAGGACATTACGAGGGGCAAAGGTTT GGTCGACTCCCTTTTCCAAGCACCAATGGGAGATGGCACGCACGAAGCA GTCCTTAGCTCCTACGAGTACCTCTCCCAGGGCTTGAAGATGTTGGACA ACACTATGGGGGGCTTCTACATCGCACCTGCTTTCATGGACAAGCTCGT CGTCCACATCTCGAAGAACTTCATGGCGCTCCCGAACATAAAGGTGCCA CTGATTCTCGGGATCTGGGGAGGCAAGGGTCAGGGGAAATCCTTCCAGT GTGAGCTCGTCTTCTCCAAGATGGGGATCAACCCCATCATGATGAGCGC CGGAGAACTGGAAAGCGGAAATGCCGGAGAACCAGCGAAACTGATCCGA CAGAGATACCGAGAGGCGGCTGACATCATCAAGAAGGGGAAGATGTGCT GCCTCTTCATCAACGATCTGGATGCGGGAGCTGGCCGAATGGGAGGGAC TACTCAATACACCGTCAACAACCAGATGGTGAACGCGACCCTGATGAAC ATCGCGGATAATCCCACCAACGTGCAACTCCCTGGGATGTACAACAAGG AGGACAATCCGCGAGTCCCCATCATCGTCACGGGCAATGATTTCTCGAC ACTCTACGCCCCGCTTATCCGAGATGGGAGGATGGAGAAGTTCTACTGG GCACCTACCAGAGACGATCGCGTAGGTGTCTGCAAAGGCATCTTTCGCA CTGACAACGTCCCGGATGAGGACATCGTCAAGATCGTCGACAGCTTCCC TGGCCAATCTATCGACTTTTTCGGCGCTCTACGAGCCCGTGTTTACGAT GACGAAGTGCGGAAATGGGTTTCGGATACGGGCGTGGAGAACATTGGCA AGAAACTGGTGAACTCTAGGGAAGGGCCACCTGAGTTCGAACAGCCGAA GATGACGATCGACAAGCTCATGGAGTACGGCCATATGCTCGTGAGGGAG CAGGAGAACGTTAAGCGTGTTCAACTGGCCGACAAGTACCTAAGCGAGG CAGCTTTGGGTGACGCTAACTCCGACGCCATGAAAACTGGTTCCTTCTA CGGGTAA
52	ATGGCTGCTGCCTTCTCCTCGACAGTTGGAGCACCTGCCTCTACACCGA CAAACTTCCTGGGCAAGAAGCTGAAGAAGCAAGTGACATCCGCCGTGAA CTACCACGGCAAGAGCTCCAACATAAACCGCTTCAAAGTCATGGCGAAG GAGCTCGATGAGGACAAGCAGACCGATCAGGACAAGTGGAAGGGACTTG CCTACGACATCTCCGATGACCAGCAGGACATTACGAGGGGCAAAGGTTT GGTCGACTCCCTTTTCCAAGCACCAATGGGAGATGGCACGCACGAAGCA GTCCTTAGCTCCTACGAGTACCTCTCCCAGGGCTTGAAGATGTTGGACA ACACTATGGGGGGCTTCTACATCGCACCTGCTTTCATGGACAAGCTCGT

SEQ ID NO:	Sequence
	CGTCCACATCTCGAAGAACTTCATGGCGCTCCCGAACATAAAGGTGCCA CTGATTCTCGGGATCTGGGGAGGCAAGGGTCAGGGGAAATCCTTCCAGT GTGAGCTCGTCTTCTCCAAGATGGGGATCAACCCCATCATGATGAGCGC CGGAGAACTGGAAAGCGGAAATGCCGGAGAACCAGCGAAACTGATCCGA CAGAGATACCGAGAGGCGGCTGACATCATCAAGAAGGGGAAGATGTGCT GCCTCTTCATCAACGATCTGGATGCGGGAGCTGGCCGAATGGGAGGGAC TACTCAATACACCGTCAACAACCAGATGGTGAACGCGACCCTGATGAAC ATCGCGGATAATCCCACCAACGTGCAACTCCCTGGGATGTACAACAAGG AGGACAATCCGCGAGTCCCCATCATCGTCACGGGCAATGATTTCTCGAC ACTCTACGCCCCGCTTATCCGAGATGGGAGGATGGAGAAGTTCTACTGG GCACCTACCAGAGACGATCGCGTAGGTGTCTGCAAAGGCATCTTTCGCA CTGACAACGTCCCGGATGAGGACATCGTCAAGATCGTCGACAGCTTCCC TGGCCAATCTATCGACTTTTTCGGCGCTCTACGAGCCCGTGTTTACGAT GACGAAGTGCGGAAATGGGTTTCGGATACGGGCGTGGAGAACATTGGCA AGAAACTGGTGAACTCTAGGGAAGGGCCACCTGAGTTCGAACAGCCGAA GATGACGATCGACAAGCTCATGGAGTACGGCCATATGCTCGTGAGGGAG CAGGAGAACGTTAAGCGTGTTCAACTGGCCGACAAGTACCTAAGCGAGG CAGCTTTGGGTGACGCTAACTCCGACGCCATGAAAACTGGTTCCTTCTA CGGGGCCGCGCCGAGCTCCTGA

Table 7. Promotor Sequence of Rubisco activase from *O. meridionalis*

SEQ ID NO:	Sequence
53	GCCTCCGATTGATGCTTCACCAAAAAACAATATCAACAGCAGTGCAAAA TTAGAATTTTTGTATTTTTGTGGTAACGCAAACCGGCCATCAAAGGGGA AAAACGTACAATGCTTATGTTGTATGTTAAGAGAAGTTTGTGTGGTGCC AAATGACAGTCCTAGCCTGACGGTTATCGAGAAAGCAGAATATGTGCAG GTGGCAGAGCAAAATATTTGTGGTAGTCCAACTAGAATACAATTTGCAT GCCATGCCTCATCCAAGAAGCCGGGCAACGAGAGGCAGCAAAAGGCTTT TCTGTGGTGATGCAAAATGAAGAGGTTATGTAGTAGCTGAGCTGATGAA GCAACTGGTCGCTAGCTGCCGGCCGGGAGACGAATGTGAGGCAAGGAAA GAAAAGAAAAAACAGAGAGAAAGAGTTGATCAGAAATGGGTGAATTCTG TGGTGAGGAAAGGTCAAGGAACTGAAGCCAAGAGATCCTTCCTACCTAC ACTAATACAATACTCCTAACTCGCTCACAGACTCCGATCCAGGTCCAAG TCATGCTATGCTGTGGATCGGCCGGCCGAGATTGCGCCACGTGTGCAGA ACCCAATCTTCAGCGTGTGGCCTGTGGGGGATCTGGAAGCTGATCCACA GGGAGGGAGGAGTGTGTGCCTCTCACAGCTTCCAACTTCCATGGCGACG TCCAATGCTATTGTATTATTTAAGGCCTACCGCAGCTCGGCCTCTACAC TTTGAGCAGCAGCGGCTGGCCATCATCAGTGATCCTCTACAATCATCGA CTTTCAGCAAATTAAG

[066] It will be understood that the invention also encompasses complexes comprising Rubisco and RCA, e.g. as represented by SEQ ID NOs: 39 and 40, modified RCA or RCA-derived polypeptides which confer thermostability to the complex. The RCA, modified RCA

or RCA-derived polypeptides include variants thereof, including substantially similar sequences, fragments, analogs, homologs, or mutants. The modified RCAs retain at least one function of RCA. These functions include, for example biological activities, such as activation of Rubisco or the ability to form a complex comprising Rubisco. In one embodiment, the polypeptides of the invention are at least 85%, 90%, 95%, 97%, 98% or 99% identical to the polypeptide sequence of any of SEQ ID NOs: 39 and 40. In some embodiments, the polypeptides of the invention are altered at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12,13, 14, 15, 16, 17, 18, positions corresponding to residues 54, 61, 82, 91, 111, 112, 113, 114, 115, 142, 171, 363, 382, 389, 393, 405, 406, 429 of SEQ ID NO: 38 *O. sativa* RCA. (i.e. the short RCA form) or corresponding to residues 54, 61, 82, 91, 111, 112, 113, 114, 115, 142, 171, 363, 382, 389, 393, 405, 406, 434 of the long form *O. sativa* RCA (SEQ ID NO: 43).

[067] It will be understood that the invention also encompasses nucleic acids encoding polypeptides that can form complexes comprising Rubisco and RCA, e.g. as encoded by SEQ ID NOs: 50 to 52, or encoding modified RCA or RCA-derived polypeptides which confer thermostability to the complex. In one embodiment, the nucleic acids encoding polypeptides of the invention are at least 85%, 90%, 95%, 97%, 98% or 99% identical to the nucleic acid sequence of any of SEQ ID NOs: 50 to 52.

[068] In another embodiment, polypeptides of the invention include fragments of RCA optionally having one or more of the above alterations. These fragments retain at least one function of RCA and are at least 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400 or 425 contiguous amino acids in length.

[069] In another embodiment the RCA polypeptide of the invention comprises SEQ ID NO: 39 or 40.

[070] In one embodiment, RCA polypeptide of the invention is a fragment that corresponds to a functional domain of RCA including, but not limited to, the ATP binding domain and the domain(s) that interacts with Rubisco. In some embodiments, polypeptides of the invention include domains of RCA such as the central ATPase domain (the AAA+ module), or at least one of the N-terminal P-loop NTPase alpha-beta-alpha subdomain or the C-terminal all-alpha subdomain. In some embodiments polypeptides of the invention include the C-terminal domain and/or the N-terminal domain of RCA. In the *O. australiensis* alpha RCA sequence, the delineations of the domains are as follows: N-terminal = 1-55 residues; AAA+ motif = 56-344; ATPase core = 181-281; C-terminal = 345-464. These delineations are based on the 3D structure of tobacco RCA (Stotz, M. et al. (2011) Nat.

Struct. Mol. Biol. 18, 1366-1370) and the amino acid sequence alignment between Oa alpha RCA and tobacco RCA.

[071] One or more residue substitutions, additions and/or deletions may be introduced into a wild-type RCA. For example 1, 2, 3, or between 4-10 or between 10-20 substitutions can be introduced and/or for example 1, 2, 3, or between 4-10 or between 10-20 additions can be introduced and/or for example 1, 2, 3, or between 4-10 or between 10-20 deletions can be introduced. For example, RCA polypeptides are generated to enhance a desirable characteristic (e.g. thermostability or the ability to form a complex with Rubisco). Alternatively RCA polypeptides may be generated to reduce an undesirable characteristic (e.g. a tendency to aggregate). In one embodiment, RCA polypeptides have improved thermostability over wild-type RCA. In another embodiment, RCA polypeptides of the invention have enzymatic activity under heat stress conditions (up to 40ºC) that is similar to or higher than the enzyme activity of same RCA under normal conditions (e.g. 30°C).

[072] Sequence alterations can be introduced by standard techniques such as directed molecular evolution techniques (e.g. DNA shuffling methods), oligonucleotide-directed mutagenesis, chemical mutagenesis, cassette mutagenesis, saturation mutagenesis and gene editing. In some embodiments sequence alterations may lead to one or more conservative or non-conservative amino acid substitutions at targeted sites. In other embodiments random mutagenesis is used to generate RCA nucleic acid molecules.

[073] A 'non-conservative amino acid substitution' is a substitution in which the amino acid residue is substituted for an amino acid residue with a dissimilar side chain. Amino acid residues with similar side chains are known in the art. For example there are amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid, asparagine, glutamine), uncharged polar side chains (e.g., glycine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

[074] Alternatively or in addition to non-conservative amino acid residue substitutions, sequence alterations may lead to conservative amino acid substitutions. A 'conservative amino acid substitution' is one in which the amino acid residue is substituted for an amino acid residue having a similar side chain. Following mutagenesis, the encoded protein can be expressed and the activity of the protein can be determined as described below.

[075] In one embodiment, the nucleic acid molecules are at least 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% identical to any of the nucleic acid molecules of SEQ ID NOs: 41, 42, 50, 51 or 52.

[076] In one embodiment, the nucleic acid molecules are the nucleic acid molecules of SEQ ID NOs: 41, 42, 50, 51 or 52.

[077] In one embodiment the nucleic acid molecule comprises SEQ ID NO: 51.

[078] In another embodiment, the nucleic acid molecules encode a polypeptide that has at least one function of RCA (e.g., Rubisco activation) and are at least 100, 250, 500, 750, 1000 or 1250 contiguous nucleotides in length.

[079] In one embodiment, a nucleic acid molecule of the invention corresponds to a nucleic acid molecule that encodes a functional domain of RCA for example the ATP binding domain and the Rubisco binding domain.

[080] The invention also provides nucleic acid molecules, chimeric genes and vectors comprising nucleic acid molecules of the invention.

[081] The invention further provides cells, preferably plant cells, comprising a nucleic acid of the present invention.

Thermostable Complexes Comprising RCA

[082] The present invention provides thermostable complexes comprising RCA and Rubisco. As used herein, the term 'improved thermostability' refers to the increased ability of a complex comprising RCA of the invention and Rubisco to function under heat stress conditions as compared to a complex comprising *O. sativa* RCA and Rubisco. In one embodiment complexes comprising RCA polypeptides of the invention have enzymatic activity under heat stress conditions (e.g. 35°C or higher) that is greater than the enzymatic activity of complexes comprising *O. sativa* under the same conditions. In another embodiment, complexes comprising RCA polypeptides of the invention have enzymatic activity under heat stress conditions (about 28°C to about 30°C, or about 30°C to about 32°C, or about 32°C to about 34°C, or about 34°C or about 36°C, or about 34°C to about 36°C, or about 36°C to about 38°C, or to about 38°C to about 40°C, or about 40°C or higher) that is substantially similar to or higher than the activity of complexes comprising *O. sativa* RCA under the same conditions.

[083] In one embodiment the invention provides a thermostable complex comprising an isolated RCA from a first plant species and an isolated Rubisco from a second plant species. For example the complex may comprise an isolated RCA from *O. australiensis* and

an isolated Rubisco from *O. sativa*. For example the complex may comprise an isolated RCA from one *Oryza* sp. and an isolated Rubisco from another *Oryza* sp. It is envisaged that the RCA polypeptides of the invention can be used to form complexes with Rubisco from any plant species, such as other monocot species. In some embodiments the Rubisco in the complex is from barley, maize, tomato, potato, rice, soybean, cotton, sunflower, alfalfa, lettuce, oilseed rape, sorghum, or wheat plants. In particular embodiments the Rubisco in the complex is from barley, sorghum, rice or wheat.

[084] The complexes may be formed *in vitro* or *in vivo.* For example the complexes may be formed *in vitro* by contacting recombinant *O. australiensis* RCA with Rubisco from *O. sativa* present in a leaf extract or Rubisco that has been purified from *O. sativa*. Alternatively, the complexes may be formed *in vivo* by expression of an RCA polypeptide of the invention in a plant such that it forms a complex with the endogenous Rubisco of the plant.

[085] Any method known in the art can be used to assay the activity of complexes comprising RCA polypeptides (including, but not limited to, Rubisco activation and ATP hydrolysis).

[086] In some embodiments, complexes comprising RCA polypeptides are assayed *in vitro*. In one embodiment, the complexes, either assembled from isolated components (e.g. those generated by recombinant expression) or complexes purified or extracted from for example, leaf tissue, can be assayed for activation of Rubisco when incubated in a solution comprising deactivated Rubisco, RuBP, ATP, and a source of labeled carbon (e.g., $[^{14}C]NaHCO_3$). In this embodiment incorporation of ^{14}C into 3-phosphoglyceric acid can be monitored to assess Rubisco activation. In another embodiment, RCA polypeptides can be assayed for ATP hydrolysis when incubated in a solution comprising ATP. The assays are typically conducted at various temperatures ranging from normal conditions to heat stress conditions.

[087] In other embodiments, complexes comprising RCA polypeptides are assayed *in vivo*. Plants which express one or more RCA polypeptides of the invention can be assayed for photosynthesis rate, rate of biomass increase, growth rates, and seed yield under heat stress conditions and compared with plants that express a wild type RCA. The plants may be grown entirely under heat stress conditions or for periods of time under heat stress conditions. In some embodiment heat stress conditions are determined at the leaf surface. Assays for photosynthesis rate, rate of biomass increase, growth rates, and seed yield are known in the art. For example, photosynthesis rates can be measured by analyzing CO_2

fixation. Growth rates may be assessed by measuring the increase in leaf area over time. Seed yield may be assessed by determining seed number and/or seed weight.

Methods of Enhancing Heat Tolerance In Plants

[088] RCA activates Rubisco in plants and is the rate limiting step especially in heat stress conditions of the photosynthetic process (Salvucci, M. E. & Crafts-Brandner, S. J. (2004) Physiol. Plant. 120, 179-186). In the absence of RCA activity, Rubisco remains inactive and photosynthesis slows or stops. The present inventors have shown that complexes comprising RCA and Rubisco lose activity at high temperatures thereby suggesting that increased temperatures destabilize and/or denature complexes comprising RCA and Rubisco thereby rendering RCA less able or unable to activate Rubisco. Thus the heat tolerance of plants can be increased by the use of complexes comprising RCA polypeptides of the present invention. In particular RCA polypeptides of the invention can be expressed in plants.

[089] Any method known in the art can be used to express at least one RCA polypeptide in a plant. For example, transgenic plants can be made to express one or more RCA polypeptides in all tissues or in a subset of tissues, preferably those tissues or organelles involved in photosynthesis. An RCA polypeptide may be expressed constitutively or be under the control of an inducible promoter, such as a heat inducible or chemical inducible promoter. In some embodiments, the expression and/or activity of the endogenous RCA of the plant may be reduced or eliminated.

Recombinant Expression

[090] Nucleic acid molecules and polypeptides of the invention can be expressed recombinantly using standard recombinant DNA and molecular cloning techniques that are well known in the art (e.g., Sambrook, Fritsch, and Maniatis, Molecular Cloning: A Laboratory Manual; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, (1989)). Additionally, recombinant DNA techniques known in the art may be used to create nucleic acid constructs suitable for use in making transgenic plants.

[091] The invention also provides chimeric genes or nucleic acids expressing chimeric proteins or polypeptides, comprising a nucleic acid molecule of the invention, or a variant thereof. The term 'chimeric gene' refers to a nucleic acid molecule formed through the combination of portions of one or more nucleic acids of different origin. A chimeric gene is an artificial gene constructed by operably linking fragments of unrelated genes or other nucleic acid sequences. In other words “chimeric gene” denotes a gene which is not normally found in a plant species, i.e. it is heterologous with respect to the plant in which it

is introduced, or refers to any gene in which the promoter or one or more other regulatory regions of the gene are not associated in nature with a part or all of the transcribed nucleic acid, i.e. are heterologous with respect to the transcribed nucleic acid. More particularly, a chimeric gene is an artificial, i.e. non-naturally occurring, gene produced by an operable linkage of a nucleic acid sequence to be transcribed.

[092] The invention also provides vectors, preferably expression vectors, comprising a nucleic acid molecule of the invention, or a variant thereof. The term 'vector' refers to a polynucleotide capable of being used to maintain another nucleic acid to which it has been linked. For example a typical vector is a plasmid or a virus.

[093] The invention provides recombinant expression vectors and chimeric genes or nucleic acid molecules that comprise a nucleic acid molecule encoding an RCA polypeptide of the invention in a form suitable for expression in a host cell. It will be understood by a skilled person that the choice of components of the chimeric gene and expression vector will depend on such factors as the host cell to be transformed, the level of expression of polypeptide desired, the tissue within the organism in which expression is desired, etc. Typically, recombinant expression vectors or chimeric genes can be introduced into host cells to produce proteins or peptides, including fusion proteins or peptides.

[094] The recombinant expression vectors or chimeric genes or nucleic acid molecules can be for expression of an RCA polypeptide in prokaryotic (e.g. bacteria or yeast) or eukaryotic cells (e.g. insect, mammalian or plant cells). Alternatively, the recombinant expression vectors can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

[095] In some embodiments, a nucleic acid molecule of the invention can be expressed in plant cells using a recombinant expression vector or chimeric gene or nucleic acid molecule designed for use in plants, for example, tobacco mosaic virus and potato virus expression vectors or any other plant expression vector known in the art.

[096] A number of promoters known in the art can be used to express the nucleic acids of the invention for example the promoters may be constitutive, tissue-specific or inducible.

[097] A 'tissue-specific promoter' may direct expression of nucleic acids of the present invention in a specific tissue, organ or cell type. A tissue-specific promoter may also be an inducible promoter. Alternatively a 'constitutive promoter' is defined as a promoter which will direct expression of a gene in all tissues and are active under most environmental conditions and states of development or cell differentiation.

[098] The term 'inducible promoter' refers to a promoter that is under precise environmental or developmental control. In particular embodiments the inducible promoter may be a chemically-inducible promoter or a heat-inducible promoter or a light-inducible promoter. Examples of chemically-inducible promoters in plants include promoters which are inducible upon exposure to chemical reagents which can be applied to the plant, such as herbicides or antibiotics. For example, the maize In2-2 promoter, activated by benzenesulfonamide herbicide safeners, can be used (De Veylder (1997) Plant Cell Physiol. 38:568-577); application of different herbicide safeners induces distinct gene expression patterns, including expression in the root, hydathodes, and the shoot apical meristem. Coding sequence can be under the control of, e.g., a tetracycline-inducible promoter, e.g. , as described with transgenic tobacco plants containing the *Avena sativa* L. (oat) arginine decarboxylase gene (Masgrau (1997) Plant J. 11 :465-473); or, a salicylic acid-responsive element (Stange (1997) Plant J. 11:1315-1324). Using chemically (e.g. hormone or pesticide)-induced promoters, i.e., promoters responsive to a chemical which can be applied to the transgenic plant in the field, expression of a polypeptide of the invention can be induced at a particular stage of development of the plant. Use may also be made of the estrogen-inducible expression system as described in US patent 6,784,340 and Zuo et al. (2000, Plant J. 24: 265-273) to drive the expression of the nucleic acids used to practice the invention.

[099] Examples of heat-inducible promoters in plants include the heat shock promoters for example the promoter of *Hsp90p* or other heat-inducible promoters such as those for genes *OsHsfB2cp* and *PM19p*.

[0100] Examples of light-inducible promoters include e.g. green-tissue promoters like the pea rbcS-3A promoter (Kuhlemeier et al. (1989) Plant Cell 1: 471-478), and the maize rbcS promoter (Schaffher and Sheen (1991) Plant Cell 3: 997-1012).

[0101] One aspect of the invention pertains to host cells into which a nucleic acid encoding an RCA polypeptide of the invention has been introduced. Introduction of the nucleic acid into the cell can be by any means known in the art, for example using microinjection; Agrobacterium-mediated transformation; direct gene transfer; ballistic particle acceleration; virus-mediated transformation; chloroplast transformation or electroporation. The term 'host cell' refers to the particular subject cell and to the progeny or potential progeny of such a cell. Modifications may occur in subsequent generations of a host cell for example due to either mutation or environmental influences, and thus the subsequent generations may not, in fact, be identical to the original host cell, but are still included within the scope of the term as used herein.

[0102] In other embodiments a nucleic acid of the invention may be introduced into a plant or plant cell by crossing a plant containing the nucleic acid with another plant that doesn't contain the nucleic acid so that at least some of the progeny of the crossing contain the nucleic acid.

[0103] Accordingly, the invention provides plants and plant cells comprising a nucleic acid of the invention.

Production of Plants

[0104] The RCA polypeptides and nucleic acids of the invention may be useful for producing transgenic plants. Any method known in the art can be used for transforming a plant or plant cell with a nucleic acid molecule of the present invention. Nucleic acid molecules can be incorporated into plant DNA (e.g., genomic DNA or chloroplast DNA) or be maintained without insertion into the plant DNA (e.g., through the use of artificial chromosomes). Suitable methods of introducing nucleic acid molecules into plant cells include microinjection; Agrobacterium-mediated transformation; direct gene transfer; ballistic particle acceleration; virus-mediated transformation; chloroplast transformation; and electroporation. The choice of transformation method used will vary depending on the type of plant or plant cell (e.g. monocot or dicot) to be transformed.

[0105] Transformed plant cells which are derived by any of the above transformation techniques can be cultured to regenerate a whole plant which possesses the transformed genotype and thus the desired phenotype. Such regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker that has been introduced together with the desired nucleotide sequences. Plant regeneration from cultured protoplasts is described in the art. Regeneration can also be achieved from plant callus, explants, organs, or parts thereof. Regeneration techniques are also known in the art.

[0106] In some embodiments the plant cells of the invention are non-propagating plant cells.

[0107] Plants comprising RCA polypeptides and nucleic acids of the invention may be produced by crossing a plant containing the nucleic acid with another plant that doesn't contain the nucleic acid so that at least some of the progeny of the crossing contain the nucleic acid.

[0108] As used herein the term 'plant' refers to whole plants, shoot vegetative organs/structures (e.g. leaves and stems), roots, flowers and floral organs/structures (e.g. bracts, sepals, petals, stamens, carpels, anthers and ovules), seed (including embryo,

endosperm, and seed coat) and fruit (the mature ovary), plant tissue (e.g. vascular tissue, ground tissue, and the like) and cells (e.g. guard cells). The term 'plant' also refers to higher and lower plants amenable to transformation techniques, including angiosperms (monocotyledonous and dicotyledonous plants), gymnosperms, ferns, and multicellular algae. Plants of a variety of ploidy levels, including aneuploid, polyploid, diploid, haploid and hemizygous plants are also included.

[0109] The nucleic acid molecules of the invention can be used to express an RCA polypeptides of the invention in any plant, including species from the genera Agrotis, Allium, Ananas, Anacardium, Apium, Arachis, Asparagus, Athamantha, Atropa, Avena, Bambusa, Beta, Brassica, Bromus, Browaalia, Camellia, Cannabis, Carica, Ceratonia. Cicer, Chenopodium, Chicorium, Citrus, Citrullus, Capsicum, Carthamus, Cocos, Coffea, Coix, Cucumis, Cucurbita, Cynodon, Dactylis, Datura, Daucus, Dianthus, Digitalis, Dioscorea, Elaeis, Eliusine, Euphorbia, Festuca, Ficus, Fragaria, Geranium, Glycine, Graminae, Gossypium, Helianthus, Heterocallis, Hevea, Hibiscus, Hordeum, Hyoscyamus, Ipomoea, Lactuca, Lathyrus, Lens, Lilium, Linum, Lolium, Lotus, Lupinus, Lycopersicon, Macadamia, Macrophylla, Malus, Mangifera, Manihot, Majorana, Medicago, Musa, Narcissus, Nemesia, Nicotiana, Onobrychis, Olea, Olyreae, Oryza, Panicum, Panicum, Panieum, Pannisetum, Pennisetum, Petunia, Pelargonium, Persea, Pharoideae, Phaseolus, Phleum, Picea, Poa, Pinus, Pistachia, Pisum, Populus, Pseudotsuga, Pyrus, Prunus, Pseutotsuga, Psidium, Quercus, Ranunculus, Raphanus, Ribes, Ricinus, Rhododendron, Rosa, Saccharum, Salpiglossis, Secale, Senecio, Setaria, Sequoia, Sinapis, Solanum, Sorghum, Stenotaphrum, Theobromus, Trigonella, Trifolium, Trigonella, Triticum, Tsuga, Tulipa, Vicia, Vitis, Vigna, and Zea.

[0110] In some embodiments the transgenic plants are barley, maize, tomato, potato, rice, soybean, cotton, sunflower, alfalfa, lettuce, oilseed rape, sorghum, or wheat plants. In particular embodiments the transgenic plants are monocots plants or cereal plants such as barley, sorghum, rice, corn or wheat. A skilled person will recognize that after production of a transgenic plant, the nucleic acid molecule of the invention in that transgenic plant can be introduced into other plants by sexual crossing. Any standard breeding technique known in the art can be used to achieve this.

Determination of Expression in Plants

[0111] Any method known in the art can be used for determining the presence or level of expression in a plant of a nucleic acid molecule of the invention or an RCA polypeptide of the invention. For example, the expression level in a plant of a RCA polypeptide can be

assessed using molecular techniques including, for example, immunoassay, immunoprecipitation, gel electrophoresis, and mass spectrometry.

[0112] Additionally, the expression of an RCA polypeptide in a plant can be assessed by a change in the plant phenotype (for example photosynthesis rate, growth rate or seed yield) under heat stress conditions compared with plants expressing wild type RCA.

[0113] Furthermore, extracts or polypeptides isolated from transgenic plants, their tissues cells or seeds can be used in *in vitro* assays.

[0114] It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

EXAMPLES

Example 1: DNA sequencing and sequence alignment

[0115] Initially total RNA was extracted from 100 mg of leaf tissue using an RNeasy Plant Mini Kit (Qiagen). RNA was converted to cDNA using a SuperScript VILO cDNA Synthesis Kit (Invitrogen) using 2.1 µg of RNA as a template. PCR was performed on 2 µl of the cDNA product using GoTaq Green Master Mix (Promega). Refer to Supplementary Methods in Scafaro, A. P., Haynes, P. A. & Atwell, B. J. (2010) J. Exp. Bot. 61, 191-202 for the PCR cycle program and the primers used. The product was run on a 1.5% agarose gel stained with Gel Red (Biotium). The bands where cut from the gel using a scalpel and the DNA purified using a DNA Gel Extraction Kit (Qiagen). The PCR products were sequenced in both directions using the same primers as PCR amplification. Sequence preparation was performed using Big Dye Terminator V3.1 sequencing reaction mix as per manufacturer's instructions and samples run on a 3130x 1 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Multiple DNA and protein sequences were aligned using the MUSCLE algorithm [Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32: 1792-1797] and the open-source software program Unipro UGENE v1.9.0.

Results: RCA and its expression

[0116] The RCA genes of *Oryza sativa* L. ssp. japonica cv. Amaroo, the two *O. meridionalis* accessions and *O. australiensis* were sequenced and differences were found between the species. There was no difference in sequence between the two *O. meridionalis* accessions. There were 50 base changes between *O. australiensis* and *O. sativa* including 44 base substitutions and six deletions in the *O. australiensis* gene. Three of the *O. australiensis* base substitutions not found in *O. sativa* were also found in *O. meridionalis*. Another four base changes were unique to *O. meridionalis*. When the DNA sequences were translated to amino acid sequence the DNA sequence differences between *O. sativa* and *O. meridionalis* did not translate into a difference in protein. There were however 18 residue changes/deletions unique to both RCA isoforms of *O. australiensis* (see Table 1[a]). The protein sequence of O. australiensis was then compared with two warm-adapted species, *Gossypium hirsutum* (cotton) and *Larrea tridentata* (creosote bush), and the cold adapted species *Spinacia oleracea* (spinach) and *Deschampsia antarctica* (Antarctic hairgrass). Four of the residue differences found in *O. australiensis* was the same as one or both of the corresponding residues found in the warm-adapted species but not in either of the cold-adapted species (see Table 1[b]).

Example 2: Superior tolerance to heat stress in the wild rice *Oryza australiensis* due to thermostable RCA.

MATERIALS AND METHODS

Plant growth - short-term heat treatment (reference Figures 1-3)

[0117] Seeds of *Oryza sativa* L. ssp. japonica cv. Amaroo (Os-A) and *O. sativa* L. ssp. indica cv. Doongara (Os-D) were obtained from the Yanco Agricultural Institute (Department of Primary Industries, NSW, Australia). Seeds *of Oryza meridionalis* Ng. were collected from wild populations located in the Cape York Peninsula of Australia at S15°42', E145°03' (henceforth referred to as *O. meridionalis* CY or Om-CY) and from Keep River north of Western Australia at S15°58', E129°03' (henceforth referred to as *O. meridionalis* KR or Om-KR). *Oryza australiensis* Domin. were collected from the same location as *O. meridionalis* KR. For all experiments, plants were grown in controlled growth cabinets (Thermoline Scientific Equipment, Smithfield, NSW, Australia) under a 12-h photoperiod with a photosynthetic photon flux density (PPFD) of 600 to 800 µmol m^{-2} s^{-1}. Plants were grown at 28/22°C (day/night) in a 3:1, soil of fine-textured krasnozem (locally sourced from Robertson, NSW, Australia) and an organic mix, in pots with no drainage holes. For leaf

elongation rate experiments, plants were grown in 2-L pots and measurements were taken at the 5th-leaf stage. For all other experiments, plants were measured after growing for 60 days in 10-L pots. Soil was kept wet at all times and allowed to pool at the surface once the plants were over 40-days old. A commercial liquid fertilizer (Aquasol, Yates, Australia) was applied once a week following manufacturer's instructions, beginning 10 days after germination.

Plant growth - long-term heat treatment (reference Figure 25-26)

[0118] Seeds of *Oryza sativa* L. ssp. japonica cv. Amaroo (Os-A) and *Oryza australiensis* Domin. were grown as described above and 7 days after germination, seedlings were exposed to three temperatures (25, 35 and 45°C) during the day (12 h) and 21 degrees at night until plants were 35 days old. Seedlings were harvested weekly by measuring leaf area, and fresh and dry biomass for shoots and roots. Relative growth rates and net assimilation rates were calculated using dry mass and leaf area for the treatment period (7-35 d after germination).

Leaf elongation rates

[0119] Leaf elongation rates were measured using a HR4000 Linear Variable Displacement Transducer (LVDT). After emergence of the fifth leaf, plants were transferred to a cabinet containing the LVDT unit and randomly assigned to one of the eight measurement stations. The fifth leaf was clipped to the apparatus and growth was measured for a 24-h period. Plants were grown at 22°C for the initial 12 h of growth in the dark followed by 28°C for the first 4 h of the light period. The cabinet temperature was then raised to 45°C for 4 h and returned to 28°C for the final 4 h of the day period. Leaf length was measured and logged every 6 mins using the software program VuGrowth, version 1.0 (Applied Measurement, Oakleigh, Victoria, Australia). The ambient temperature of the cabinet was logged ever 10 min by a portable data logger (Onset HOBO, Massachusetts, USA) placed next to the plants. Leaf lengths were converted to leaf elongation rate (LER) by subtracting the leaf length at any given time by the leaf length 1 h prior to that measurement. The experiment was repeated on six occasions with nine plants from each genotype measured in total.

Gas-exchange and Rubisco activation state assays

[0120] Gas-exchanges were measured using a Licor 6400 (LI-COR, Lincoln, USA). The CO_2 in the reference chamber air was set at 380 ppm and a PPFD of 1500 µmol m^{-2} s^{-1} was used for all measurements. Measurements were made in the growth cabinet and the air temperature of the gas exchange cuvette was set to 28 or 45°C to match the cabinet temperature. Control measurements were made at 28°C beginning 2 h into the light period.

Measurements at 45°C commenced after 2 h of exposure to 45°C and continued over a 2 h period by randomly measuring the different genotypes over that 2 h period. All measurements were made on healthy, fully-expanded leaves. Measurements were made on 3-6 leaves from 3 pot replicates. To determine the activation state of Rubisco, two 0.266 cm^2 leaf disks were rapidly excised from mature fully-expanded leaves using a leaf punch and immediately frozen in liquid nitrogen. The Rubisco activation state was calculated by measuring total and initial Rubisco activities. Samples were collected throughout the light period to coincide with growth measurements. Samples were collected after 2 h at 28°C (2 h light), 2 h at 45°C (4 h light), 4 h at 45°C (6 h light) and again at 28°C after 1 h of recovery from the higher temperatures (7 h light). Four replicates were collected from separate pots placed randomly throughout the cabinet.

Immunoblot analysis

[0121] For immunoblot analysis, plant material was harvested at a cabinet temperature of 28°C or after 2 h at 45°C as described above for Rubisco activation. Plant material was ground in liquid nitrogen and 100 mg of fine tissue was weighed before extraction by vortexing in 800 µl of SDS extraction buffer containing 30% sucrose, 2% SDS, 0.1 M Tris-HCl, pH 8.0, 5% β-mercaptoethanol. Samples were centrifuged at 10,000 g for 5 min and 10 µl of the supernatant was added to 3 µl of 5X SDS-loading dye (Invitrogen). Samples were electrophoresed in NuPage BIS-TRIS 6-12% gradient SDS-PAGE gels (Invitrogen) together with pre-stained molecular weight markers (Precision Plus All Blue Standard, Bio-Rad, city, country). Polypeptides were transferred to polyvinylidene difluoride (PVDF) membranes for immunological detection of RCA using an anti-tobacco activase primary antibody (Law, D., Crafts-Brandner, S. & Salvucci, M. (2001) Planta 214, 117-125.) in conjunction with an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Sigma Aldrich). Immunoblots were stained with NBT/BCIP (Promega). Immunoblot analyses were conducted in triplicate, i.e., using samples prepared from three different plants. RCA primary antibody used in western blot analysis has a greater affinity for RCA-Oa than RCA-Os. Taking into consideration the difference in antibody reactivity through a standard curve generated from purified recombinant RCA of known concentration, the concentration of RCA in the leaf was obtained.

Statistics

[0122] All graphs and statistics were created using GraphPad Prism 5.0d software (GraphPad Prism Software Inc., San Diego, USA). For both LER and Rubisco activation state data, a two-way ANOVA was run initially. When appropriate, this test was followed by individual one-way ANOVA and Tukey's multiple comparison tests. Gas-exchange data was

analysed with a two-sample t-test between control and heat treatment for each genotype independently.

RESULTS

Temperature-dependent growth and photosynthesis

[0123] The relative heat sensitivity of four genotypes was determined by comparing their LER to the LER of Os-A (Figure 1). The comparison showed that the LERs of all genotypes were similar to that of Os-A at 28°C, but at 45°C the rates were comparatively higher, exhibiting species-specific differences in the response to high temperature. As the temperature was lowered to 28°C, LER was again similar amongst all the genotypes, including Os-A. Rates of leaf elongation were similar for all of the rice genotypes after a 1-h exposure to 45°C. However, by the end of a 4-h heat exposure period, differences in the growth rates between Os-A and the four other genotypes were obvious. *O. sativa* cv. Doongara (Os-D) was more tolerant than Os-A, but less tolerant than the wild rice genotypes. The two *O. meridionalis* populations (Om-CY and Om-KR) had a similar response and their heat tolerance was intermediate between Os-D and O. australiensis. *Oryza australiensis* showed the greatest tolerance to high temperature; after 4-h of exposure to 45°C its LER was double that of Os-A.

[0124] A significant interaction between the effects of growth temperature and exposure time on LER was observed for all the genotypes, except for *O. australiensis* (Table 8). This interaction was evident by the relatively stable growth rates at 28°C compared with reduced rates at 45°C (Figure 2). *Oryza australiensis* was unique among the genotypes in maintaining a similar LER at 28 and 45°C (Figure 2e). Because of the lack of growth inhibition at 45°C, there were no significant differences in the LER between the two temperatures or over the exposure period for *O. australiensis*. When comparing the LER over the 4-h exposure period at 45°C, all five genotypes exhibited the highest rates at the lowest exposure time (1-h). For both *O. sativa* cultivars, the LER continuously and markedly decreased over the 4-h exposure time (Figures 2a, b). A more moderate decrease in LER occurred in the two *O. meridionalis* populations (Figures 2c, d), whereas the LER of *O. australiensis* was not significantly affected over the 4-h period of exposure to 45°C.

Table 8. Results of a two-way ANOVA for the data shown in Figure 2 with P-values given for comparisons between the two growth temperatures, the exposure time at each temperature and the interaction between the two variables.

	P-values		
Genotype	**Temperature**	**Exposure time**	**Interaction**
O. sativa cv. Amaroo	0.1477	0.0197	<0.001
O. sativa cv. Doongara	0.9734	0.0050	<0.001
O. meridionalis CY	0.0382	0.0277	<0.001
O. meridionalis KR	0.6711	0.8775	<0.001
O. australiensis	0.2373	0.6785	0.1331

Plants were grown at an ambient temperature of either 28 or 45°C for an exposure time of 4 h and leaf lengths measured every 6 min using an LVDT (refer to Figure 1). All statistics were based on six experimental replicates.

[0125] Heat applied to plants during daylight hours for four weeks was used to test whether the heat tolerance effects seen for leaf elongation in *O. australiensis* could be replicated over longer periods (Figure 25). Two main effects were observed: (1) *O. australiensis* grew as fast at 45°C as at 35°C and (2) growth in *O. sativa* slowed dramatically after three weeks of heat treatment. Relative growth rates reflected this, with *O. australiensis* shoots about one-third the biomass of those of *O. sativa* by the end of the heat treatment. Figure 26 shows the correlation between relative growth rate and net assimilation rate/specific leaf area, the two determinants of relative growth rate. This illustrates that net assimilation rate is the driving variable for growth across six temperature × genotype combinations. This is turn suggests that metabolism and photosynthesis/respiration are the key drivers of growth, not leaf construction.

[0126] The effect of elevated temperature on net photosynthetic rate (P_n) was also species-specific. Net photosynthesis decreased after 2-4 h at 45°C for all the genotypes, except *O. australiensis* (Figure 3). The decrease in P_n was more marked for *O. sativa* (42% for Os-A and 39% for Os-D), intermediate for *O. meridionalis* (25% for Om-CY and 29% for Om-KR) and minimal for *O. australiensis* (1%).

Rubisco activation state

[0127] The response of Rubisco activation state to increased temperature (Table 9) was similar to the response observed for LER and P_n in the five genotypes (Fig. 2 and 3). Upon exposure to 45°C, Rubisco activation decreased the most in the two *O. sativa* cultivars, compared with a more moderate decrease in the two *O. meridionalis* populations and a minimal decrease in *O. australiensis*. For all five genotypes, there was an initial decrease in the activation state of Rubisco after 2 h at 45°C. Interestingly, the decrease in Rubisco activation that occurred after a 2-h exposure to 45ºC was lowest in the two genotypes from the hottest environment, i.e., Keep River. When the exposure to heat was extended to 4 h, Rubisco activation either remained at the same lower level (Os-A and Om-KR & -CY) or decreased further (Os-D) except in *O. australiensis*. In *O. australiensis*, Rubisco activation recovered during the extended exposure to 45°C and when a 1-h recovery period at 28°C followed the 4-h heat exposure, achieving an activation state comparable to the initial activation state at 28°C. In contrast, the activation state of Rubisco in the other two species remained inhibited even after a 1-h recovery period at 28ºC.

[0128] To determine whether there was a significant difference in the response of Rubisco activation state to exposure time at high temperature among the various genotypes, a two-way analysis of variance was performed. The analysis indicated that there was no significant genotype × exposure time interaction ($F = 0.9516$, $P=0.5037$, $df =12$). One-way ANOVA revealed significant differences ($P = 0.0016$) between species only after exposure of the plants for 4 h at 45°C, when the activation state of Os-A was lower than *O. australiensis* and the activation state of Os-D was lower than both Om-KR and *O. australiensis* (Table 8). There were no significant differences ($P>0.05$) in activation state between the *O. meridionalis* and *O. australiensis* genotypes. Moreover, there was no significant effect ($P>0.05$) of the elevated temperature on the activation state for these three genotypes. Conversely, both *O. sativa* cultivars exhibited a significant decrease ($P = 0.0032$ for Os-A and $P = 0.002$ for Os-D) in activation state after a 4-h exposure to 45°C compared with the activation state at 28°C.

Table 9. Rubisco activation state (%) of five rice genotypes in response to changes in ambient temperature.

Temperature	***O. sativa***		***O. meridionalis***		***O. australiensis***	**P-value**
	cv. Amaroo	cv. Doongara	CY	KR		
28°C	64 ± 1	70 ± 8	72 ± 2	68 ± 5	68 ± 7	0.8540
2 h at 45°C	44 ± 5	47 ± 9	47 ± 4	55 ± 6	54 ± 9	0.7453
4 h at 45°C	42 ± 6	27 ± 3	46 ± 7	53 ± 6	67 ± 4	0.0016
28°C recovery	29 ± 3	30 ± 2	46 ± 12	44 ± 8	66 ± 14	0.0670
P-value	0.0032	0.0022	0.1439	0.1761	0.6866	

A one-way ANOVA was used to determine differences between genotypes at a single growth condition and another one-way ANOVA for differences within a genotype across all growth conditions. Plants were analysed before (28°C), during the heat treatment (2 h at 45°C and 4 h at 45°C) and also after a recovery period of 1 h at 28°C (28°C recovery). All values are means ± SE (n = 4).

[0129] The similarity in the temperature response of LER and Rubisco activation state for each genotype led to the determination of a positive relationship between the two variables (Figure 4). Linear regression analysis indicated that 51% of the change in LER could be explained by changes in Rubisco activation state.

RCA and its expression

[0130] Sequencing of the RCA genes from *O. sativa* cv. Amaroo, the two O. *meridionalis* populations and *O. australiensis* revealed differences in RCA sequence between the three species, but not between the two *O. meridionalis* populations. There were 50 base differences between *O. australiensis* and *O. sativa* including 44 base substitutions and six deletions in the *O. australiensis* gene. Three of the base substitutions found in *O. australiensis* when compared to *O. sativa* were also found in *O. meridionalis*. In addition, another four base changes were unique to *O. meridionalis*. When the DNA sequences of the RCA genes were translated to protein sequences, the differences between *O. sativa* and *O. meridionalis* at the nucleotide level did not translate into differences at the amino acid level. In contrast, comparison of the amino acid sequences for RCA from the different genotypes revealed three residue deletions and 15 residue substitutions that were unique to *O. australiensis, for both the small form and the large form*.

[0131] For all five rice genotypes, the abundance of RCA protein was not affected by exposure to 45°C for 2 h, nor was the ratio of the large to small form of RCA. Irrespective of temperature there was a similar total abundance of RCA between all genotypes.

Conclusions

[0132] Three rice species differed in their heat tolerance, as indicated by differences in vegetative growth at 45°C. Domesticated rice, *O. sativa*, was the most susceptible to inhibition by heat, whereas the wild rice *O. meridionalis* was less susceptible and *O. australiensis* exhibited tolerance to heat stress. The variation in tolerance among the species was related to the level of photosynthetic inhibition at the elevated temperature, which in turn was related to the degree to which Rubisco deactivated. Heat-tolerant *O. australiensis* exhibited no reduction in growth, photosynthesis or Rubisco activation when exposed to 45°C for over 2 h. To account for the differences in thermotolerance, we propose that differences in the structure of activase between *O. australiensis* and the other two species, coupled with a potentially greater abundance of the protein, ameliorate the thermotolerance of Rubisco activation in *O. australiensis* with a consequent effect on photosynthesis and growth.

Example 3: In planta Rubisco and RCA stoichiometry

[0133] Rubisco and RCA concentration was determined by Western Blot analysis of extracted total soluble leaf protein calibrated against purified Rubisco and RCA of known concentration.

[0134] To determine *in vivo* concentrations of the two enzymes, standard curves were generated using purified enzyme of known concentration. The concentration of Rubisco was determined using an antibody specific for the 53 kDa large subunit of Rubisco. Because the quaternary structure of the Rubisco complex comprises 8 large and 8 small (16 kDa) subunits, the total Rubisco content could be deduced from these westerns. Table 4 lists the concentrations of Rubisco and RCA for the two species and the ratio between the two functionally complete enzymes. While there was substantial variation between biological and technical replicates using this indirect method, there was no evidence for statistically differences between the species. However, in the absence of a more precise technique that is relatively simple, we believe that these concentrations are credible. Despite some variation, the *in vivo* enzyme concentrations we report in Table 10 match the concentrations found after protein purification (i.e. concentrations achieved when extracting RCA and Rubisco from leaves). Considering the concentration of Rubisco and RCA were determined from the same tissue samples we can be relatively confident in the comparative ratios of the

two proteins across species. However differences in the absolute concentration of Rubisco and RCA between the species may be affected by differences in growing conditions between harvests, particularly nitrogen availability, which is influenced by life-stage.

Table 10. Rubisco and RCA *in vivo* concentrations

	Rubisco		**RCA**		**Rubisco:RCA**	
	(mg.g^{-1} FW#)	**(uM*)**	**(mg.g^{-1} FW)**	**(µM)**	**(g:g)**	**(M:M)**
O. sativa	5.7±1.2	10.3±2.2	1.3±0.6	5.9±2.7	4.4:1	1.7:1
O. australiensis	6.8±2.2	12.3±4.0	1.8±0.5	8.2±2.3	3.8:1	1.5:1

Milligrams of protein per gram of leaf fresh weight, based on Western blot analysis.
* The molarity is based on the functional Rubisco enzyme molecular weight being 553,000 Da and the RCA oligomer with most activity having a molecular weight of 220,000 Da based on native gel analysis (presented in Example 4). One gram of fresh weight is assumed to equate to 1 mL of volume. Both *O. sativa* and *O. australiensis* were grown at 28ºC.

Example 4: RCA concentration-dependent oligomer formation (native gels)

[0135] Native gel analysis has allowed for the concentration-dependent self-oligomerisation of RCA subunits to be observed. To the inventors' knowledge this is the first time native gels have been used to visualise the interaction of RCA subunits. Others have shown similar RCA self-assembly in cotton and tobacco using fluorescence and small angle X-ray scattering techniques (Henderson, J. N., Hazra, S., Dunkle, A. M., Salvucci, M. E. & Wachter, R. M. (2013) Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics 1834, 87-97; Wachter, R. et al. (2013) Photosynthesis Res. 117, 557-566.). Similarly, *O. australiensis* (Oa) and *O. sativa* (Os) RCA form a larger complex through self-association when the enzyme concentration is increased. The inhibition of activation activity at low RCA concentrations is a result of the self-associating complex falling below a certain threshold of about three RCA subunits. Note that due to differences in protein isoelectric points and protein physical shape, native bands may migrate ±15% from what is expected based on molecular weight alone.

[0136] Rather than being distinct in size, increasing concentration of RCA led to a range of RCA oligomer sizes, shifting from lower to higher molecular weights as the concentration increased. With increasing concentration, the beta isoform seems to form at least three distinct oligomer molecular weights while the alpha isoform is found as mostly a single oligomer that shifts to higher molecular weights with increased RCA concentration.

[0137] Although there was distinct RCA complex and Rubisco complex bands on native gels there was no discernible Rubisco/RCA complex when the two enzymes were mixed.

[0138] To establish temperature effects on the protein complexes of interest, samples were incubated at 28°C or 38°C for 20 minutes before loading on native gels. The higher temperature did not interfere with the individual Rubisco or RCA complexes. This supports the conclusion that while the Rubisco complexes and RCA complexes from Oa and Os exhibit different thermostability characteristics, each is stable at high temperatures and the interaction of the Rubisco with RCA (leaf extracted or recombinant), i.e. the Rubisco-RCA complex, is heat-labile, particularly in *O. sativa* (see Figure 21).

[0139] SDS-PAGE separation of leaf-purified RCA from *O. australiensis* and from wheat. Total leaf protein was initially precipitated in 35% saturated ammonium sulphate followed by anion-exchange chromatography using Q-sepharose media and varying salt concentrations. The beta isoform eluted from anion-exchange at 0.2 M KCl while the alpha isoform eluted at 0.5 M KCl. The 0.2 M fraction containing RCA was used in subsequent assays of RCA interaction with wheat Rubisco.

[0140] Bands of interest from the native gels were excised and LC-MS/MS was run on trypsin-digested proteins extracted from those bands. Identified proteins are listed in Table 11. Protein identification was as expected with the clear identification of the two Rubisco subunits and identification of RCA ranging in molecular weight. Two proteins identified in some of the leaf-extracted RCA bands were glutamine synthase and sucrose synthase. It is unlikely that an interaction between RCA and glutamine or sucrose synthase is occurring, rather it is more likely that these proteins form complexes of similar molecular weight to RCA, therefore occupying the same band-space. Note that only the beta isoform of RCA was identified in all bands associated with RCA, however considering that the two isoforms are identical apart for 38 amino-acids at the C-terminus of the alpha isoform, it is likely that both were present but the peptide sequences had a higher probability of matching the identical regions and assigning the peptide to the beta rather than alpha isoform.

Table 11.

Spot Number	Protein Identification	Peptide Matches#
1	Rubisco large subunit Rubisco small subunit	30 5
2	Rubisco large subunit Rubisco small subunit	22 6
3	Peroxisomal hydroxyl-acid oxidase	1
4	RCA beta isoform Glutamine synthase	21 5
5	RCA beta isoform	2
6	RCA beta isoform Glutamine synthase	6 1
7	RCA beta isoform Sucrose synthase	4 2

.

Refers to the total number of peptides that matched the identified protein.

Example 5: Concentration-dependent activity of RCA

[0141] Figure 5 shows the concentration-dependent Rubisco activation rate of leaf-extracted RCA from *O. australiensis* and *O. sativa*, as well as Os (solid lines) and Oa (dashed lines) alpha and beta recombinant isoforms. The Rubisco activation assay was performed essentially as described by Barta, C., Carmo-Silva, A. E. & Salvucci, M. E. (2011) Methods Mol. Biol. 684, 375-382, with two modifications: (1) the quenched second-stage assay samples were dried on a heating block before resuspension in scintillation cocktail prior to counting; (2) all reported Rubisco activation assays were of inhibited Rubisco incubated with RCA for 5 min prior to determining Rubisco activity. Recombinant RCA was produced as described in Barta, C., Carmo-Silva, A. E. & Salvucci, M. E. (2011) Methods Mol. Biol. 684, 363-374. The following plasmids were used: pEV61, pGA220, pEV63 and pGA221 encoding *O. sativa* RCA alpha and beta isoforms and *O. australiensis* alpha and beta isoforms respectively. As well as giving the absolute RCA concentrations used in assays, the molar ratio of RCA to Rubisco is also given, as the activation reaction is dependent on the concentration of Rubisco as well as RCA. Oa-leaf RCA can reach high rates of activity relative to Os RCA when high concentrations of RCA with a molar ratio of RCA to Rubisco of 1:6 were used. Under these conditions, rates of activation are increased relative to those for the recombinant form of RCA. This molar ratio is well above what is

found in the leaf and marked in Figure 5 by the vertical lines. It is unlikely that the low *in vitro* specific activity of leaf-extracted RCA from Oa would be reflected *in vivo* because photosynthetic rates between the two species are similar, which would not be the case if Oa RCA was functioning at only 40% of Os rates. The reduced in vitro specific activity in Oa is therefore likely to be related to *in vitro* measurements for a reason yet unknown. Interestingly, Os leaf RCA also has a lower specific activity when compared to its recombinant counterpart but the difference is minimal).

[0142] If the lower specific activity seen in RCA extracted from Oa leaf were to be caused by a non-specific inhibitory substance, it would be expected that such a substance would also inhibit recombinant RCA. To test this we ran a mixing experiment where Os leaf extract, from which RCA had been removed, was added to recombinant Oa beta RCA and RCA activity measured (Figure 6). No inhibition of the recombinant protein was observed when leaf extract, depleted in RCA, was added to it.

Example 6: RCA heat-stability

[0143] To determine the heat-stability of RCA three techniques were used. Two techniques, the NADH coupled assay and the phosphate assay measure the hydrolysis of ATP by RCA, which is a function the enzyme performs irrespective of the presence of Rubisco but is specifically necessary for Rubisco activation. The third technique, the Rubisco activation assay, directly measures the ability of RCA to activate Rubisco by assessing CO_2 binding. All three techniques require RCA to be purified, at least partially. For Rubisco activation assays, Rubisco as well as RCA must be purified prior to experimentation. Experiments used either partially purified RCA from leaf extracts or complete purification of recombinant protein expressed in *E. coli*.

Example 7: RCA recombinant gene expression and protein purification

[0144] The transformed *E. coli* containing the vectors pEV61 (containing the rcaIOs-2Pb gene (encoding the *O. sativa* long isoform RCA)), pEV63 (containing the rcaIOau-2Pb gene (encoding the *O. australiensis* long isoform RCA)), pGA220 (containing the rcaIIOs-3Pb gene (encoding the *O. sativa* short isoform RCA)) and pGA221 (containing the rcaIIOau-3Pb gene (encoding the *O. australiensis* short isoform RCA)) for expression of the *O. sativa* and *O. australiensis* and isoforms of RCA. The plasmid maps of these vectors are given in Figure 7. The expression and purification of RCA allowed for analysis of the heat-stability of the enzyme. As the expression vector allowed for relatively high concentrations of the

protein to be produced, the protein could be highly purified. Contrastingly, lower concentrations of RCA extracted from leaves did not allow for leaf-extracted RCA to be purified to the same extent as dilution during gel filtration chromatography led to the loss of the RCA complex and subsequently an inability to purify the protein to a high degree.

[0145] All of the vectors except pGA221 expressed RCA and for every 100 ml of cell culture approximately 2.5 mg of protein was purified. The purification of recombinant RCA involved precipitation of the RCA by adding saturated ammonium sulphate to the homogenised *E. coli* cell extract. Precipitated RCA was then suspended in buffer and loaded onto a gel-filtration column and the relevant peak collected. A second chromatographic step of passing the RCA through a strong anion-exchange column provided the final purified RCA. Running the purified protein on a gel showed the extent of the purification, with almost no other proteins visible.

Example 8: RCA from leaf extracts and partial purification

[0146] Unlike purification of the recombinant protein, leaf-extracted RCA was more challenging to work with, which is why rice RCA has never been successfully analysed *in vitro*. A couple of attempts to purify leaf extracted RCA in a similar manner to purification of the recombinant protein failed. The challenge with leaf-extracted RCA is the limited amount of RCA available per unit of leaf and the presence of proteases which quickly degrade the protein. Partial purification of RCA from leaf extract was possible, which involved precipitating out RCA with saturated ammonium sulphate, followed by desalting. While RCA was by no means the only protein left in the sample, it was the most abundant and gels indicated that there was little degradation by proteases. As RCA is the only protein responsible for activation of Rubisco, the presence of other proteins in the sample has little effect on the ^{14}C-Rubisco activation assays. Activation assays performed on leaf-extracted RCA samples are useful as the impact of post-translational modifications and protein-protein interactions on the activity of RCA are at least partly accommodated. In other words, this assay is more representative of what can be expected *in vivo* in transgenic plants.

[0147] SDS-PAGE Gel image analysis of the partially purified leaf extracts show *O. australiensis* had a lower content of RCA, which was factored into some Rubisco activation results. However, the concentration of RCA in relation to partially purified protein extracted from the leaf was similar between *O. sativa* and *O. australiensis*, being about 25% of the partially purified protein for both species. Similar concentrations of RCA between the species observed in leaf extracts is highly relevant to gene expression and protein abundance.

[0148] Gels showed *O. sativa* to have a greater abundance of the isoform relative *to O. australiensis*, with *O. sativa* having about equal quantities of the two isoforms while *O. australiensis* has much more β than α isoform. The ratio of the two isoforms seems to change with growth temperature. In particular, *O. sativa* grown for 72 hours at a raised day temperature of 40°C had almost no visible β form but substantially more α form relative to plants grown at a day temperature of 30°C.

Example 9: Rubisco purification

[0149] For the Rubisco activation assay purified Rubisco, the substrate in the assay, is also required. One gram of leaf fresh weight yielded approximately one milligram of Rubisco. Purification of Rubisco involves extraction from leaves followed by precipitation of the protein with saturated ammonium sulphate. Following ammonium sulphate precipitation the sample was passed through a sucrose gradient using high-speed centrifugation with the large Rubisco complex migrating to higher concentrations of sucrose. Following sucrose gradient separation the sample was separated by anion-exchange chromatography resulting in a highly pure extract of Rubisco.

Example 10: ATPase assays

[0150] RCA requires ATP to activate Rubisco. The ATPase activity of RCA is not directly related to the Rubisco activation activity, as Rubisco is not required for ATP hydrolysis to occur. The ATPase activity of RCA from both species (Oa and Os) was measured over a range of temperatures. Two alternative methods were used to determine ATPase activity. Firstly, a coupled reaction involving the conversion of NADH to NAD^+ and the subsequent change in absorbance at 340 nm was measured (Figure 8). Alternatively the production of phosphate when ATP is converted to ADP + P_i was measured (Figure 9). A similar temperature response was observed between the two methods.

[0151] Results from both ATPase activity based assays show that, irrespective of species, the β isoform has a substantially higher specific activity than the α isoform.

[0152] To compare the temperature response between the two methods used to determine ATP hydrolysis (the phosphate and NADH coupled reaction), normalised values attained from both methods were plotted together (Figure 10).

Example 11: ^{14}C Rubisco activation assays

[0153] A two-stage assay in which RCA is initially incubated with inactivated Rubisco for a set period of time, followed by measurements of Rubisco specific activity, allows for determination of the efficiency of RCA activation of Rubisco. For all of the experiments, inactivated Rubisco was incubated with RCA for a period of five minutes at given temperatures. The total activity of Rubisco, independent of RCA, was determined by removing Rubisco from the incubation mix allowing for all Rubisco sites to become active. Of note, at temperatures as high as 42°C the total activity did not fall but increased slightly. The absence of a reduction in total Rubisco activity with increasing temperature supports the consensus view that RCA is heat labile while Rubisco is relatively insensitive to high temperature (Salvucci, M. E. & Crafts-Brandner, S. J. (2004) Plant Physiol. 134, 1460-1470; Crafts-Brandner, S. J. & Salvucci, M. E. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 13430-13435.) .

[0154] Two independent sets of experiments measuring the activity of leaf-extracted RCA were undertaken. Figure 11 relates to RCA extracted from leaf material. RCA was extracted from leaf material of three occasions and results are based on three biological replicates and up to eight technical replicates. Measurements were made at 25, 30, 34, 36, 38, 40 and 42°C. The activation values have not been adjusted for differences in RCA concentration between the two species, as the amount of purified protein extract added to each assay was unknown. The change in activation with temperature is therefore more informative than the absolute values given (Figure 11a).

The values were subsequently adjusted for RCA concentration differences between the two species (Figure 11b).

[0155] Rubisco activation shows RCA_{Os} to be more active at a temperature of 30°C but the activity of RCA_{Os} declines sharply at temperatures of 36°C and above. Contrastingly, RCA_{Oa} is less active at moderate temperatures but the enzyme maintains close to a constant rate up to temperatures of 42°C. Analysis of individually plotted curves give a peak in RCA activation performance of 29.9 ± 0.5 °C for *O. sativa* and 33.0 ± 0.3 °C for *O. australiensis*.

[0156] After differences in RCA protein concentration between the species are accounted for, activation at 36°C is not significantly different between the species (as indicated in Figure 11b). Furthermore, at higher temperatures of 38 and 42°C RCA_{Oa} activity is greater. When protein adjusted values are normalised to 30°C the tolerance of RCA_{Oa} activity at temperatures above 38°C is even more apparent (see Figure 11c).

[0157] Analysis of Rubisco activation by the recombinant purified protein was also undertaken (Figure 12). The recombinant protein was measured at 30 and 36°C. Interpretations from previous work have led to an expectation that a higher ratio of α to

β isoform would improve heat stability of the enzyme. However, with an increasing ratio of α isoform the activity of the RCA complex falls. The fall in activity with increased α isoform is more pronounced for *O. sativa* and more so at 30°C than at 36°C, however the pattern seems to exist for both species and temperatures. Interestingly, although the β isoform of RCA_{Os} has higher activity than RCA_{Oa} there is little difference in activity of the α isoform between the species.

[0158] Until now Oa-leaf RCA heat-stability has only been measured at a fraction activated level of ≤ 0.1 Oa-leaf RCA and has now been measured as fraction activated rates > 0.3 by increasing the concentration of extracted leaf protein and consequently RCA concentration. To evaluate whether the heat-stability of Oa-leaf RCA is maintained even when the activity matches that of Os, a temperature response curve of Rubisco activation was performed with varying concentrations of Oa leaf-extracted RCA (Figure 13).

[0159] When Rubisco activation in Oa extracts was enhanced by using enzyme concentrations comparable to those in Os extracts, RCA from the leaf of Oa exhibited superior heat-stability relative to that from Os. The highest concentration of Oa RCA measured had the same absolute rate as Os RCA at 25°C. Above 25°C the activity of Oa RCA outperformed Os RCA and at 42°C Oa had an absolute rate of activity four-times that of Os. It seems from the current results that heat-stability is slightly reduced when the concentration is as high as 0.96 $mg.mL^{-1}$, with close to a 50% drop in activity at 42°C relative to 30°C, compared with less than a 20% fall at concentrations of 0.24 and 0.49 $mg.mL^{-1}$. It may reflect a stoichiometric relationship between RCA and Rubisco. However even at this 'high' concentration, the fraction activated is still well above the 80% reduction by Os RCA at comparable temperatures.

Example 12: Thermostability of the rice RCA oligomer

[0160] Differential Scanning Fluorimetry (DSF) is a relatively new and simple way of determining the thermal stability of proteins. It relies on the signal of a dye that preferentially fluoresces in a non-polar (unfolding protein) rather than polar environment (folded or aggregated protein). DSF was performed on recombinant RCA_{Os} and RCA_{Oa} using SYPRO Orange dye and a temperature ramping of 1°C per minute from 25°C to 90°C (Figure 14). First derivative (dR) values of fluorescence were plotted against temperature, with the protein melting temperature (T_m) being the temperature at which the first derivative was at a maximum. T_m is the temperature at which there are equal concentrations of folded and unfolded protein. Figure 14 shows that all four RCA variants have a T_m ranging from 41 to 43°C. This supports the main conclusions of the ATPase activity results. Specifically, that

the RCA oligomer is heat-stable at temperatures up to 40°C and that there is little difference in the heat-stability of the RCA oligomer between the two species.

Example 13: Heat-dependent regulation of RCA isoforms

[0161] Protein abundance of the alpha and beta isoforms of RCA from heat-treated rice plants was examined and the same samples were subjected to anion-exchange chromatography and run on SDS-PAGE gels. The benefit of the anion-exchange sample preparation step is that the two isoforms of RCA can be seen more clearly. Interestingly, there is much less alpha isoform present in Oa than what looked to be present in the original ammonium sulphate precipitated samples. Possibly there was an overlapping protein that made it seem like the previous samples of Oa had a greater abundance of the alpha isoform.

[0162] Image analysis SDS-PAGE gel alongside RCA isoform gene expression shows a broadly similar pattern of response to higher growth temperatures between gene expression and protein abundance of the two Os isoforms (Figure 15). The RCA isoform gene expression analysis was performed as described by Scafaro, A. P., Haynes, P. A. & Atwell, B. J. (2010) J. Exp. Bot. 61, 191-202. With increased temperature, both gene expression and protein abundance of the Os alpha isoform increase within 24 h but level off and fall thereafter. Despite the similar response of the Os RCA isoform to increased temperature, there is a marked quantitative divergence between gene expression and protein abundance, as the alpha isoform only constitutes 30% of beta RCA mRNA but there was 50% more alpha than beta RCA protein after 24 h at 38°C. In contrast to Os, heat increased expression of the alpha isoform of Oa but did not enhance the alpha isoform of the protein product.

Example 14: Concentration-dependent ATPase specific activity (recombinant protein)

[0163] Inhibition of Rubisco activation at low RCA concentration may be attributed to either a failure of the protein to self-assemble into a functioning oligomer or to a positive allosteric interaction between RCA and Rubisco. To establish which phenomenon accounts for the loss of function at low RCA concentration, the specific activity of ATP hydrolysis by RCA was determined over a range of RCA concentrations (Figure 16). The ATPase activity of RCA is independent of Rubisco and conditional on formation of a self-aggregating oligomer. It is therefore reasonable to suggest that ATP hydrolysis by RCA is the most direct indicator of the functioning of the self-interacting enzyme oligomer. The results given in Figure 16 support the conclusion that low RCA concentrations limit oligomer formation, leading to a

loss of RCA activity. This is evident in that specific activity of ATP hydrolysis falls at low RCA concentrations. If the RCA oligomer was not inhibited by low RCA concentration the specific activity would be constant, as enzymatic concentration is implicit in the specific activity calculation. Previous studies of spinach also show that ATPase activity was inhibited by low RCA concentrations, attributed to concentration effects on the self-aggregating oligomer (Wang, Z. Y., Ramage, R. T. & Portis Jr, A. R. (1993) Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology 1202, 47-55.). Of note, the inhibition of ATPase activity at low concentration is greater for RCA_{Oa} than RCA_{Os} and more so for the alpha than beta isoform, matching the Rubisco activation concentration-dependent limitations.

Example 15: Temperature response of recombinant RCA

[0164] The temperature response of partially purified leaf-extracted RCA from the *Oryza* species shows Rubisco activation activity by RCA from *O. sativa* to be susceptible to temperatures of 36°C and above (Figure 11). Contrastingly, Rubisco activation activity by RCA from *O. australiensis* extracted from leaves is stable at all measured temperatures up to 42°C.

[0165] ATP hydrolysis activity was assayed as described by Barta, C., Carmo-Silva, A. E. & Salvucci, M. E. (2011) Methods Mol. Biol. 684, 375-382. Recombinant RCA_{Oa} has lower ATP hydrolysis activity than RCA_{Os} at comparable concentrations (Figure 9). Figure 17 plots RCA oligomer heat stability, as determined by ATPase activity, against the RCA-Rubisco complex heat stability, as determined by RCA-mediated Rubisco activation. The temperature stability of ATP hydrolysis of recombinant RCA_{Oa} matches that of RCA_{Os}, both being heat stable, not losing activity until 40°C (Figure 9), well above the inhibition of Rubisco activation activity by recombinant RCA (Figure 17). The interaction of RCA with Rubisco is therefore an important factor in heat stability of RCA activation activity, more so than the susceptibility of RCA to temperature in isolation.

Example 16: Oligomeric RCA heat stability versus RCA-Rubisco heat stability

[0166] The heat-labile nature of both *O. sativa* and *O. australiensis* recombinant RCA-Rubisco complexes as presented by the solid lines in Figure 17 raises an interesting consideration. As the self-aggregating recombinant RCA oligomer (*O. sativa* and *O. australiensis*) is heat-stable (determined by the heat stability of ATP hydrolysis, indicative of a functional protein) yet the RCA-Rubisco complex is heat-labile (determined by the Rubisco activation activity of RCA, which is reliant on RCA and Rubisco interactions), the

susceptibility of the RCA to heat in rice must therefore be due to protein-protein interactions of RCA with Rubisco rather than RCA interaction with itself. Figure 17 plots RCA oligomer heat stability, as determined by ATPase activity, against the RCA-Rubisco complex heat stability, as determined by RCA-mediated Rubisco activation. The results support RCA-Rubisco complex heat susceptibility as the limitation to Rubisco activation for both species.

Example 17: Interaction of RCA with *O. australiensis* Rubisco

[0167] Until now all Rubisco activation experiments have used Rubisco extracted and purified from *O. sativa*. Examining the interaction of RCA_{Os} and RCA_{Oa} with *O. australiensis* Rubisco is important considering the RCA-Rubisco interaction is critical to the temperature limitation in RCA activity. Figure 18 compares the temperature-dependent response of both recombinant and leaf-extracted RCA against Rubisco extracted and purified from the two species. Key findings are:

- Irrespective of the Rubisco used, recombinant RCA is heat labile.
- The heat-stability of leaf-extracted RCAs is improved when interacting with O. *australiensis* Rubisco rather than *O. sativa* Rubisco.

[0168] The results show that the primary structure of Rubisco from *O. australiensis* is different from that of *O. sativa* and Rubisco can influence the stability of the RCA-Rubisco complex. From interactions between Os and Oa leaf extracted RCA and Os and Oa Rubisco (Figure 18) it seems that a reduced Rubisco activation activity of leaf extracted RCA at ambient temperature, as compared to recombinant RCA, is associated with increased heat-stability of Rubisco activation activity of the leaf extracted RCA at higher temperature.

Example 18: Regulation of the alpha and beta isoform of RCA – heat-dependent up-regulation of the alpha isoform

[0169] With increased growth temperature from 28 to 38°C there is an increase in mRNA expression of the alpha isoform of RCA. The beta isoform expression stays relatively constant, with high growth temperatures increasing the ratio of alpha to beta RCA isoforms (Figure 19). The increase in ratio of alpha to beta isoform with heat is the same for both *O. sativa* and *O. australiensis*, suggesting a common pattern of regulation of the genes in both species. The increase in expression of the alpha isoform peaks within 72 h of heat exposure before beginning to drop slightly by 120 h of heat. After 48 hours of reducing the growth

temperature back to 28°C, the level of alpha to beta isoform mRNA returns to pre heat-treatment levels.

[0170] Protein abundance of the alpha and beta isoforms of RCA is proportional to mRNA levels. Visualisation of partially purified RCA extracts run on SDS-PAGE indicate greater alpha RCA protein abundance from 72 h of heat exposure onwards. Unlike RNA expression, there is no discernible increase in alpha isoform protein abundance before 72 h but a greater abundance at 28°C after recovery from heat exposure. The results suggest protein abundance has a 48-h lag-time behind changes in gene expression.

[0171] To test whether changes in isoform composition of the RCA complex influence the heat-stability of RCA enzymatic function, the temperature effects on Rubisco activation by RCA extracted from plants over the time-course of heat treatment were measured at 28 and 38°C and are shown in Figure 20. The heat-stability of RCA is not improved by increasing the ratio of alpha to beta isoform. In fact, RCA heat-stability falls slightly in both species when there is a greater presence of the alpha isoform.

Example 19: The ability of RCA from rice to activate wheat Rubisco

[0172] To determine the activation activity of rice RCA and wheat RCA on Rubisco from wheat ($Rubisco_{wheat}$), RCA was partially purified from leaves. The alpha and beta isoforms were separated using anion-exchange chromatography and the beta isoform was used to determine the Rubisco activation activity. The beta isoform was used as it had a higher purity of RCA and less Rubisco than the alpha isoform fraction. Although by no means pure (approx. 60-70% of total protein) the samples were of higher purity than the ammonium sulphate precipitate samples previously used (see Figures 5, 11 and 13) for leaf extracted RCA experiments (approx. 30-40%).

[0173] When comparing Rubisco from the two rice species and wheat, there was a similar response to temperature (Figure 22A). For all three species the specific activity of fully carbamylated Rubisco ranged from 0.5 to 0.8 $U.mg^{-1}$ across the measured temperature range and all had a slight increase in activity with increasing temperature. Inhibited Rubisco without the addition of RCA also had a slight increase in specific activity with increased temperature and all three species had a similar rate, never exceeding 0.2 $U.mg^{-1}$.

[0174] When $Rubisco_{wheat}$ was activated by leaf-extracted beta isoform, at moderate temperature of less than 34°C, wheat RCA was at least twice as active as the rice RCA isoforms (Figure 22B). There were smaller differences above 34°C, as a results of the stronger inhibition of $Rubisco_{wheat}$ at higher temperatures. The temperature-response curve of wheat RCA acting on $Rubisco_{wheat}$ was similar to leaf- extracted RCA from rice acting on

$Rubisco_{Os}$ (Figure 11), with a slight decline from 25 to 34°C but a more pronounced decline when temperature reached 38 and 42°C.

[0175] Panel C of Figure 22 shows recombinant forms of RCA_{Os} and RCA_{Oa} were able to activate $Rubisco_{wheat}$ to the same extent as activating Rubisco from rice ($Rubisco_{rice}$). Unlike the results from leaf extracted RCA, the results from recombinant RCA suggest that RCA from both rice species is capable of activating $Rubisco_{wheat}$ to the same extent as $Rubisco_{rice}$ and are therefore completely compatible with $Rubisco_{wheat}$.

Example 20: Growth chamber experiment with transgenic rice lines under high day temperature at 45°C

Plant expression vectors and transformation

[0176] The coding sequences for the short RCA (rcaII) and long RCA (rcaI) coding region from *O.australiensis* were obtained by chemical DNA synthesis and were adapted to rice codon usage. Different vectors were made (Fig. 7) using the synthetic genes with the complete sequence of rcaIIOa (SEQ ID NO 52) and with a 5AA truncated sequence of rcaIIOa (SEQ ID NO 51), as well as the with the complete sequence of rcaIOa (SEQ ID NO 50). In all vectors, the gene was driven by the promoter of rcaI from *O. meridionalis* (SEQ ID NO 53). The intermediate cloning vector (e.g. pTKAW39, pTKA40, pTNVS299) was constructed in *Escherichia coli*. It was transferred to the acceptor *Agrobacterium tumefaciens* strain via a heat shock. Agrobacterium-mediated gene transfer of the intermediate cloning vector(s) resulted in transfer of the DNA fragment between the T-DNA border repeats to the plant genome.

As target tissue for transformation, immature embryo or embryo-derived callus derived from japonica and indica rice cultivars which were cut into small pieces, essentially using the technique described in PCT patent publication WO 92/09696. Agrobacterium was cocultivated with the rice tissues for some days, and then removed by suitable antibiotics. Transformed rice cells were selected by addition of glufosinate ammonium (with phosphinothricin 5 mg/L) to the rice tissue culture medium.

Calli growing on media with glufosinate ammonium were transferred to regeneration medium. When plantlets with roots and shoots had developed, they were transferred to soil, and placed in the greenhouse.

Genotypes

[0177] Five transgenic lines - numbered 475 to 479, were tested. Information on the plants tested are set out in Table 12.

Table 12. List of transgenic rice events used in the high day temperature experiment.

Designation	Description	Clone Information
475	pTNVS299	Single copy line
476	pTNVS299	Single copy line
477	pTKA40	Single copy line
478	pTKAW40	Single copy line
479	pTKAW39	Single copy line
480	azygous control	NA

Experimental design

[0178] Transgenic rice (T3 homozygous rcaIIOa single copy lines) and pooled azygous lines were sown in 66-wells sowing trays after seeds had been soaked in water for 1.5 days. The seedlings were transplanted to 9 cm pots after 20 days and 14 individuals of similar size/height (visual score) were chosen for the experiment. A randomized block design with border plants was used. The growth chamber normal/control conditions were 12h light (ca. 300 μmol m^{-2} s^{-1} PAR), 22°C during the night and 28-29°C during the day. The maximum temperature was imposed for 12h during the light period while plants were held at 22°C overnight. Temperature was increased stepwise from 28/29°C to 45°C over a 48 hour period 10 days after transplanting (30 days after sowing). See Figure 23 for the temperature settings once 45°C was achieved (high day temperature). Relative humidity was kept between 60 and 70% throughout the experiment.

Leaf elongation rates (LVDT)

[0179] Leaf elongation was measured on plants every day for five consecutive days, once in the 'morning period' (about 9 am until about 1 pm), and again in the 'afternoon period', which commenced by 2 pm when plants were attached to the machine. Measurements for the 'afternoon' plants were therefore terminated the following morning. One plant of each genotype (five viable transgenic lines and one azygous control) was attached to the pulley system and readings commenced, with leaf length logged 10 times hourly. Nutrient solution was liberally supplied throughout. Plants were chosen successively from 14 blocks, with a new young emerging leaf being required for each of the ten measurements made over five days. Rates of elongation were analysed in the normal way, using logs in Excel spreadsheets to calculate rates (mm h^{-1}) over the period of steady-state elongation in the hottest period. Rates did not differ significantly between genotypes during the night.

Leaf gas exchange

[0180] After 2 weeks of high day temperature treatment, leaf gas exchange measurements were carried out during the peak temperature in the afternoon. The following parameters were recorded with an open infrared gas analyser system LI-6400XT (LI-COR Inc., Lincoln, NE, USA):

a) Net photosynthesis rates (A_n) under saturating light of 1500 PAR (integrated light source of the LI-6400XT leaf chamber) and at 400 ppm CO_2 and 45°C (block temperature setting),

b) maximum carboxylation rates of Rubisco ($V_{c,max}$), derived from the slope of A_n vs. C_i (internal sub-stomatal CO_2 concentration) curves that were recorded by stepwise changing CO_2 concentration around the leaf inside the leaf chamber,

c) the rate of A_n induction during dark-light transitions, subjecting plants to low light (ca. 50 PAR) for about 30min, then illuminating the measured leaf at 1500 PAR while following the rise of A_n over time (ca. 15min).

[0181] The initial slope was taken as surrogate for induction kinetics of photosynthesis and hence of Rubisco activation (Carmo-Silva and Salvucci, 2013 Plant Physiol. *161*, 1645–1655; Yamori et al., 2012 Plant J. Cell Mol. Biol. *71*, 871–880).

[0182] Statistical analysis (ANOVA test) was carried out with in-house developed R-package.

Flowering time / biomass / seed weight

[0183] The days to flowering are recorded for every plant. After first plant starts to flower temperature will be reduced to 28-29°C (control conditions) in order to avoid pollen sterility. All plants are harvested at full maturity and number of tillers, panicles and seeds as well as vigor (visual score 1 to 6), seed set (%), fresh and dry weight (g) per plant are taken. Seed weight is recorded after drying seeds.

RESULTS

Rubisco activation and photosynthesis

[0184] The transgenic rice plants 479 with the pTKAW39 vector showed higher values of A_n and V*c,max* than their azygous controls after 2 weeks of heat treatment (Figure 24), while the other four lines had similar A_n and a tendency towards increased V*c,max*. The rate of induction of Rubisco activity (slope A_n min^{-1}) was significantly higher in the transgenic plants 479 with the pTKAW39 vector (22%) and higher in 477 (pTKAW40) than in the azygous counterparts, indicating a beneficial effect of the short RCA variants of *O. australiensis* (rcaIIOa) on the activation of Rubisco. Thus, expression of rcaIIOa apparently contributed to a better photosynthetic performance under heat stress.

Leaf elongation rate (LER)

[0185] Six plant lines, numbered 472 to 479, and an azygous control, 480 were tested for leaf elongation rate (LER) and the results are set out in Table 13 and Table 14. These rates are means calculated across the heat period as defined in Figure 23. LERs declined by about 50% over the period of heating, reflecting increasing an inhibitory effect as heat had its effect on metabolism, but recovered to between 6 and 8 mm h^{-1} by the following morning. Lines 475 and 479 elongated fastest, with Line 475 being the only genotype to be significantly faster than the other seven genotypes, although Line 479 did not elongate significantly slower than Line 475.

Table 13. LERs for seven RCA rice transformants and an azygous control (Line 480)

Genotype designation	**Leaf elongation rate (mm h^{-1}) during heat period**
472	3.92 b
473	3.62 b
474	3.87 b
475	4.80 a
476	3.91 b
478	3.70 b
479	4.26 a,b
480	3.88 b

Tiller development

[0186] Tiller numbers were counted for plants in each block after a week of heat treatment (six-week-old plants) as a measure of shoot development (Table 14). Tiller numbers were re-counted after seven weeks of heat treatment and are reported in Figure 30. Line 479 always had significantly more tillers (7 per plant) than the others lines, including the control (Line 480).

[0187] Figure 31 shows results of tillers per plant for eight lines counted in six-week-old plants that had been exposed to one week at 45^{o}C day temperatures as described in Figure

23. Lines 1-8 are listed in the order, 474, 472, 478, 476, 473, 475, 480 (control) and 479, respectively.

Table 14. Numbers of tillers in six-week-old plants

Genotype	**479**	**475**	**480 (C)**	**476**	**478**
Block					
1	7	5	5	6	5
2	8	5	5	5	5
3	8	5	7	5	6
4	7	5	7	8	4
5	7	8	4	4	5
6	7	5	8	5	5
7	6	6	7	5	7
8	7	8	6	4	6
9	8	7	5	5	4
10	7	8	7	6	4
11	6	6	6	7	9
12	9	5	8	5	5
13	4	5	4	6	3
14	7	7	6	5	4
Average	7.0	6.1	**6.1**	5.4	5.1
S.E.M.	0.29	0.32	**0.33**	0.27	0.38

[0188] It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of technology as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive,

Claims:

1. A complex comprising an Rubisco activase (RCA) from *Oryza australiensis* and a Ribulose-1,5-bis-phosphate carboxylase/oxygenase (Rubisco) from a plant other than *O. australiensis* wherein the RCA confers thermostability to the complex.
2. The complex of claim 1, wherein the RCA comprises at least one of SEQ ID NOs: 2 to 37, 39 or 40.
3. The complex of claim 2 having at least 90%, at least 95%, at least 99% or 100% sequence identity to SEQ ID NOs: 39 or 40.
4. The complex according to any one of claims 1 to 3 being formed in a modified or transgenic plant.
5. Isolated RCA from *Oryza australiensis,* or functional variants thereof, capable of forming a complex with a Rubisco from a plant other than *O. australiensis and* conferring thermostability to the complex.
6. The RCA of claim 5 comprising at least one of SEQ ID NOs: 2 to 37, 39 or 40.
7. The RCA of claim 5 or 6 having at least 90%, at least 95%, at least 99% or 100% sequence identity to SEQ ID NO: 39 or 40.
8. An isolated nucleic acid encoding the RCA of any one of claims 5 to 7.
9. The isolated nucleic acid according to claim 8 comprising one or more of SEQ ID NOs: 41, 42, 50, 51 and 52.
10. The isolated nucleic acid according to claim 9 comprising SEQ ID NO: 51.
11. The isolated nucleic acid according to claim 9 or 10 having at least 90%, at least 95%, at least 99% or 100% sequence identity to SEQ ID NOs: 41, 42, 50, 51 and 52.
12. A chimeric gene or vector comprising the nucleic acid of any one of claims 8 to 11.
13. The chimeric gene or vector of claim 12 wherein the nucleic acid is operably linked to a promoter, preferably a plant expressible promoter.
14. The chimeric gene or vector of claim 13 wherein the promoter is a heat-inducible promoter, green-tissue promoter or a chemical-inducible promoter.
15. A host cell comprising the complex of claims 1 to 3, the RCA of any one of claims 5 to 7, the nucleic acid of any one of claims 8 to 11 or the chimeric gene or vector of any one of claims 12 to 14.
16. The host cell of claim 14, wherein the host cell is a plant cell.
17. A plant or plant cell comprising the complex of any one of claims 1 to 3, or 16 wherein the plant or plant cell is other than *O. australiensis.*

18. The plant according to claim 17 selected from the group consisting of barley, maize, tomato, potato, rice, soybean, cotton, sunflower, alfalfa, lettuce, canola (oilseed rape), sorghum, and wheat.

19. The plant according to claim 18 being rice.

20. The plant according to claim 18 being wheat.

21. A method of enhancing heat tolerance in a plant comprising expressing the RCA of any one of claims 5 to 7 in the plant.

22. The method of claim 21 further comprising providing the plant with a nucleic acid of any one of claims 7 to 10 or the chimeric gene or vector of any one of claims 11 to 13.

23. The method of claim 21 or 22 wherein the plant is capable of fixing carbon dioxide in heat stress conditions.

24. The method of any one of claims 21 to 23 wherein the RCA is expressed in the one or more plastids of the plant.

25. The method of any one of claims 21 to 24 wherein expressing the RCA results in an increase in the rate of photosynthesis under heat stress conditions.

26. Use of a complex comprising a Rubisco activase (RCA) from *Oryza australiensis* and a Ribulose-1,5-bis-phosphate carboxylase/oxygenase (Rubisco) from a plant other than *O. australiensis* to confer thermostability to the complex in a plant by the RCA.

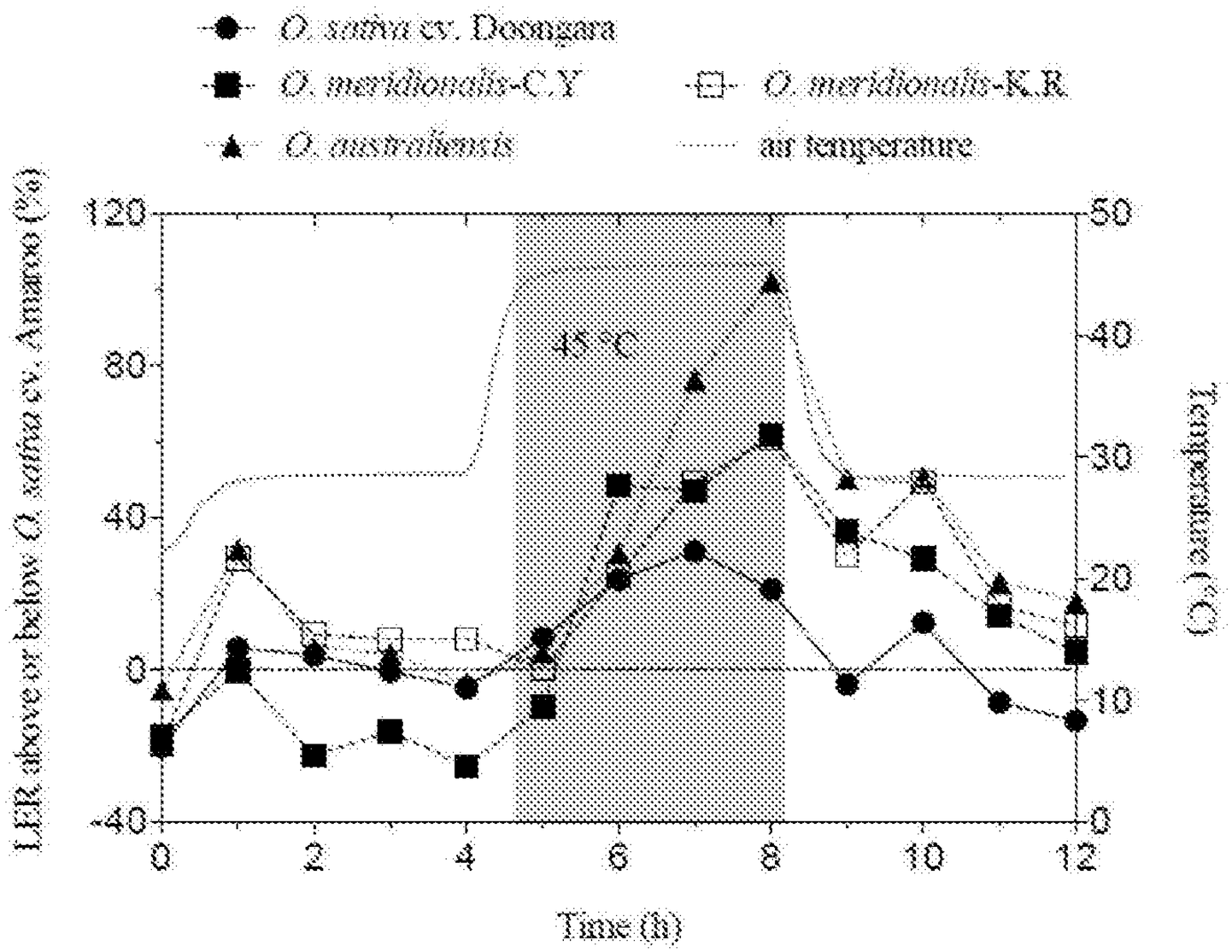

O. sativa cv. Deerngara
O. meridionalis-C.Y
O. meridionalis-K.R
O. australiensis
air temperature
45 °C
LER above or below O. sativa cv. Amaroo (%)
Temperature (°C)
Time (h)
120
80
40
0
-40
50
40
30
20
10
0
0
2
4
6
8
10
12

Figure 1

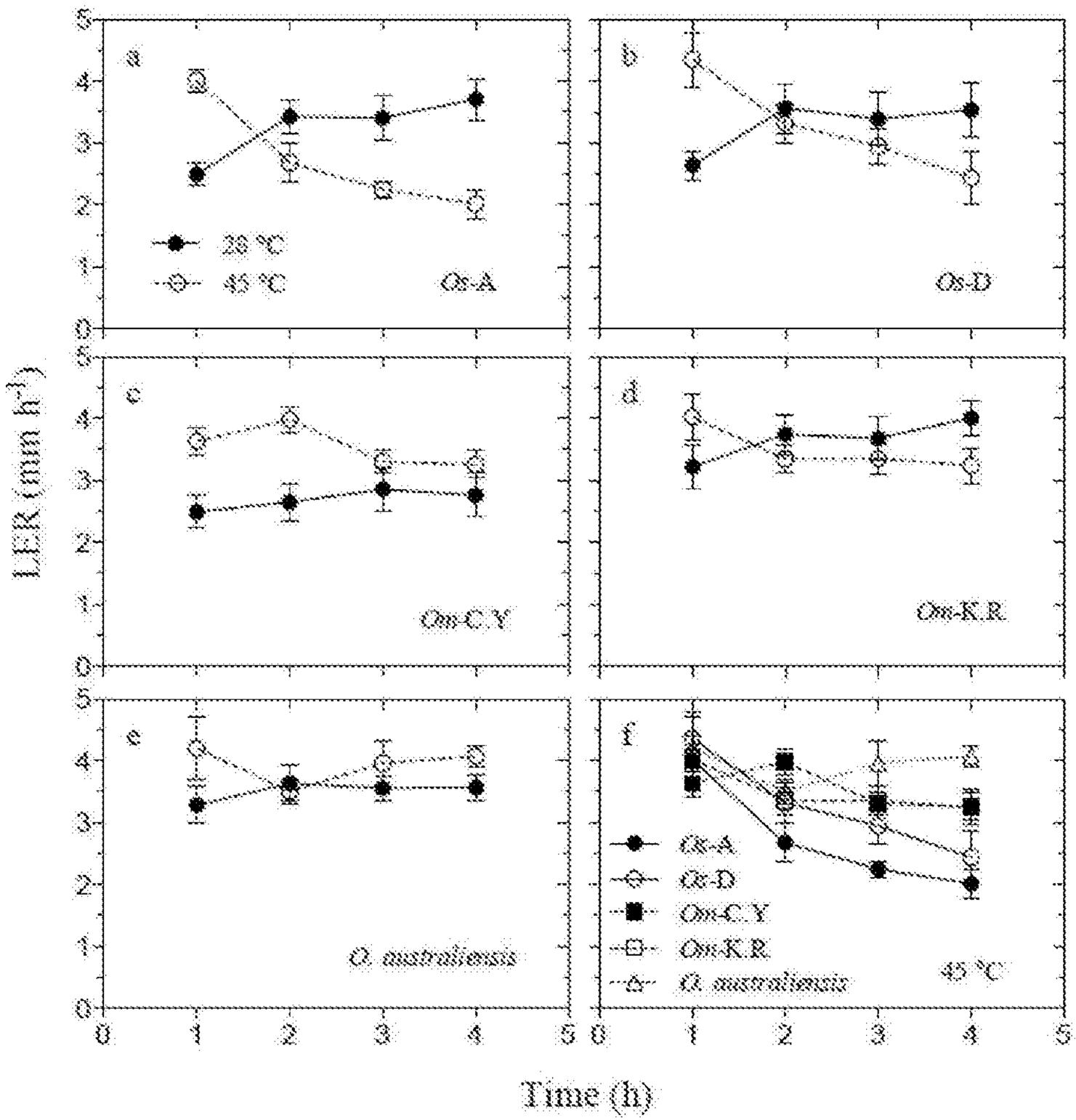

a
b
c
d
e
f
20 °C
45 °C
Os-A
Os-D
Os-C.Y
Os-K.R
O. australiensis
Or-A
Or-D
Os-C.Y
Os-K.R
O. australiensis
45 °C
LER (cm h-1)
Time (h)

Figure 2

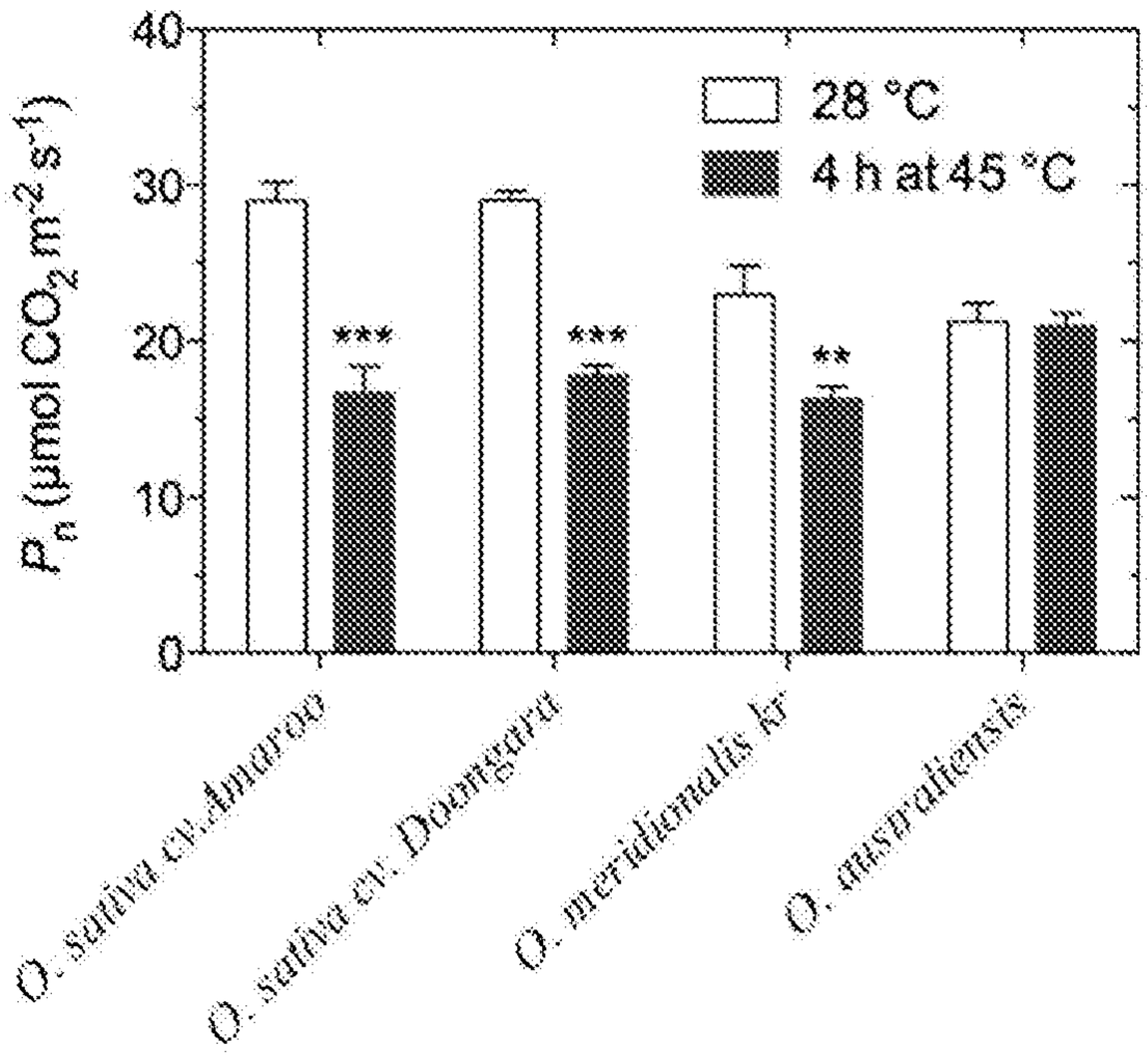


Figure 3

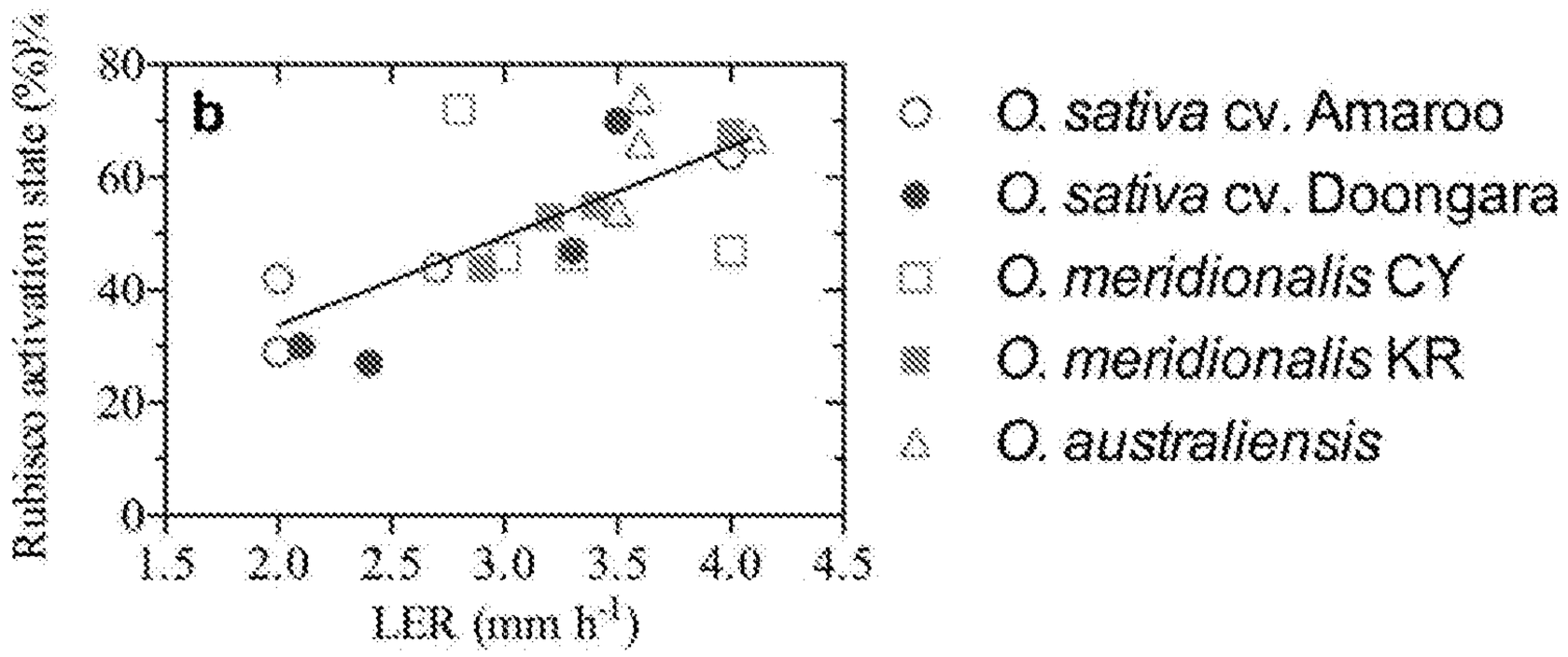
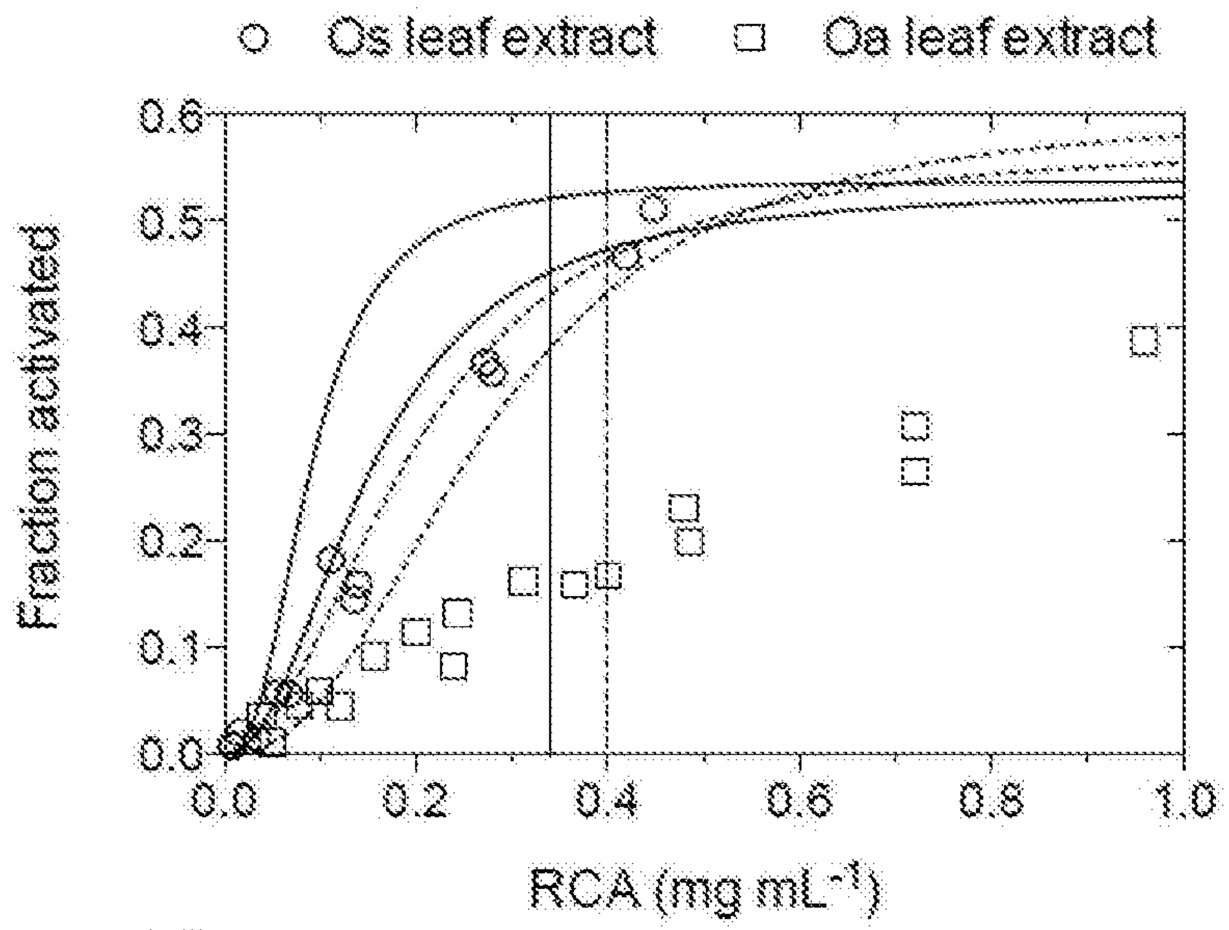
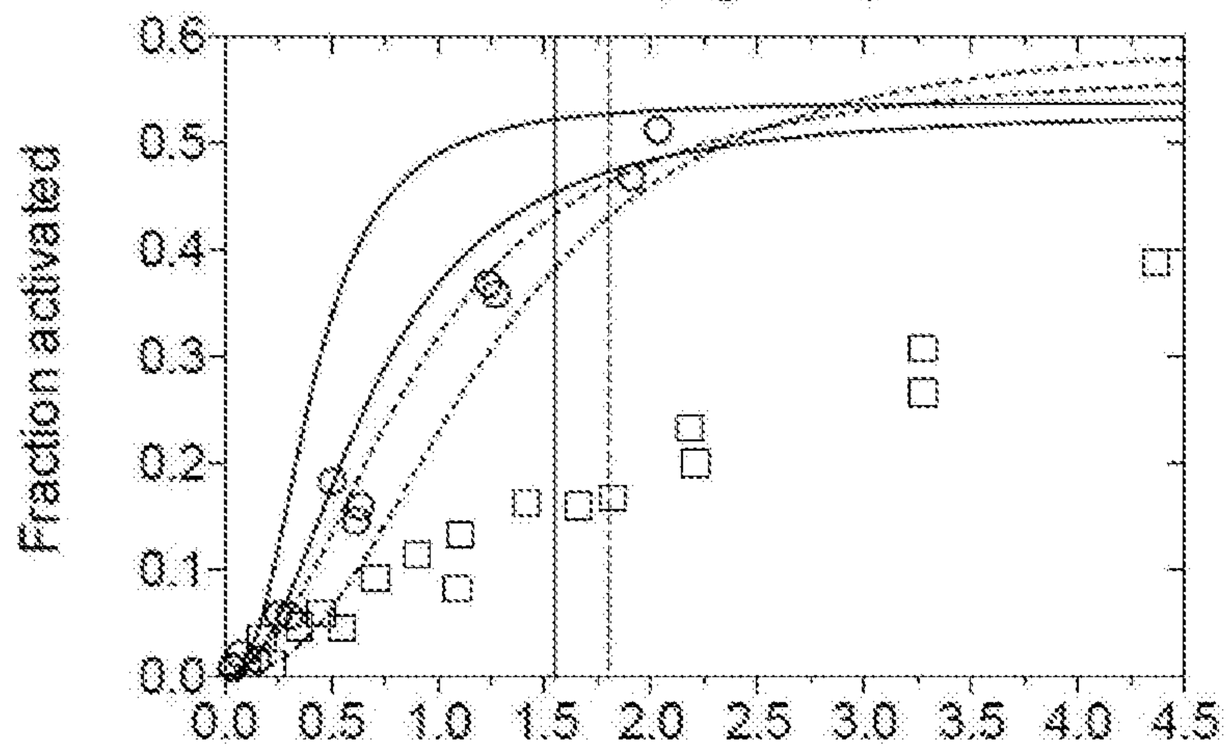


Figure 4

A


Os leaf extract
Oa leaf extract
Fraction activated
RCA (mg mL-1)

B


Fraction activated
RCA (uM)

C

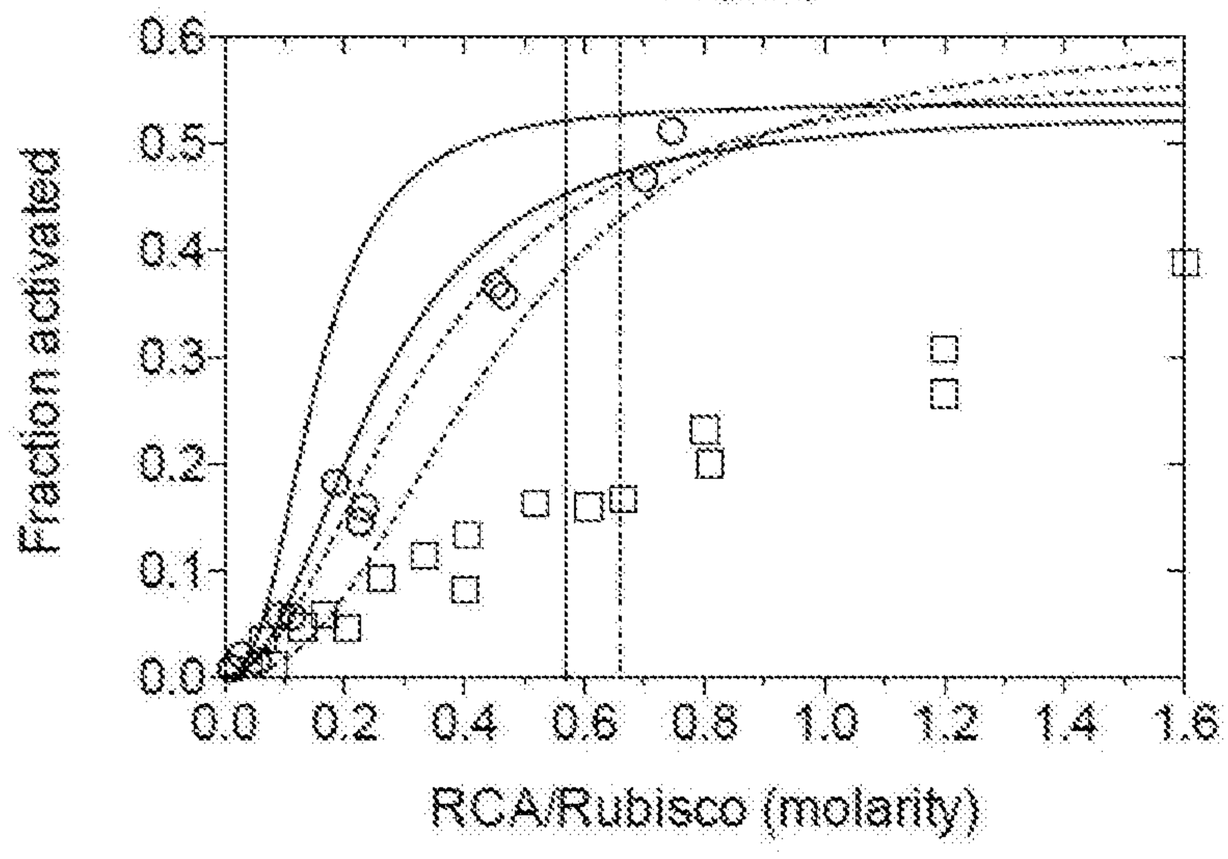

Fraction activated
RCA/Rubisco (molarity)

Figure 5

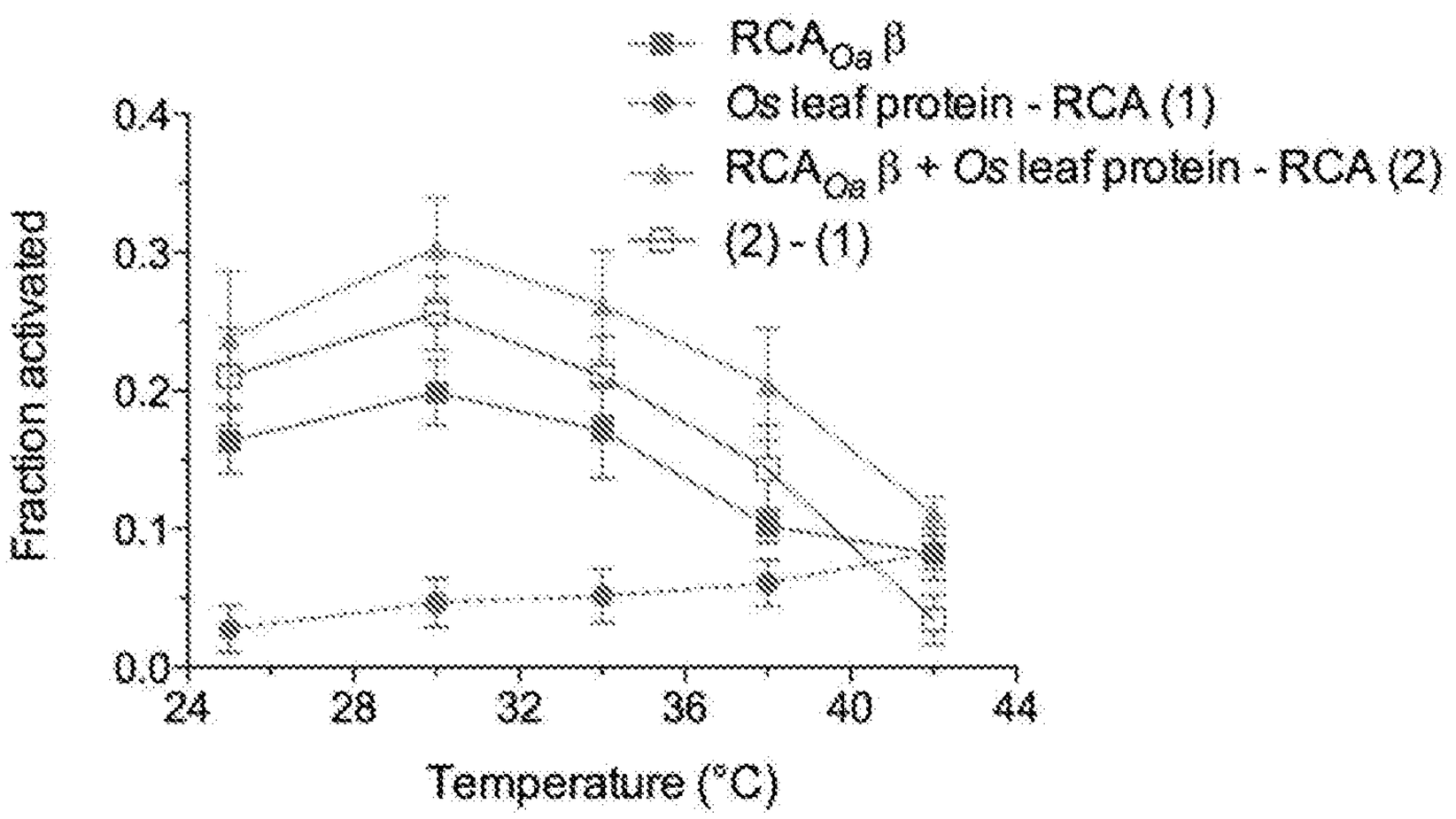

RCA$_{Os}$ β
Os leaf protein - RCA (1)
RCA$_{Os}$ β + Os leaf protein - RCA (2)
(2) - (1)
Fraction activated
0.0
0.1
0.2
0.3
0.4
24
28
32
36
40
44
Temperature (°C)

Figure 6

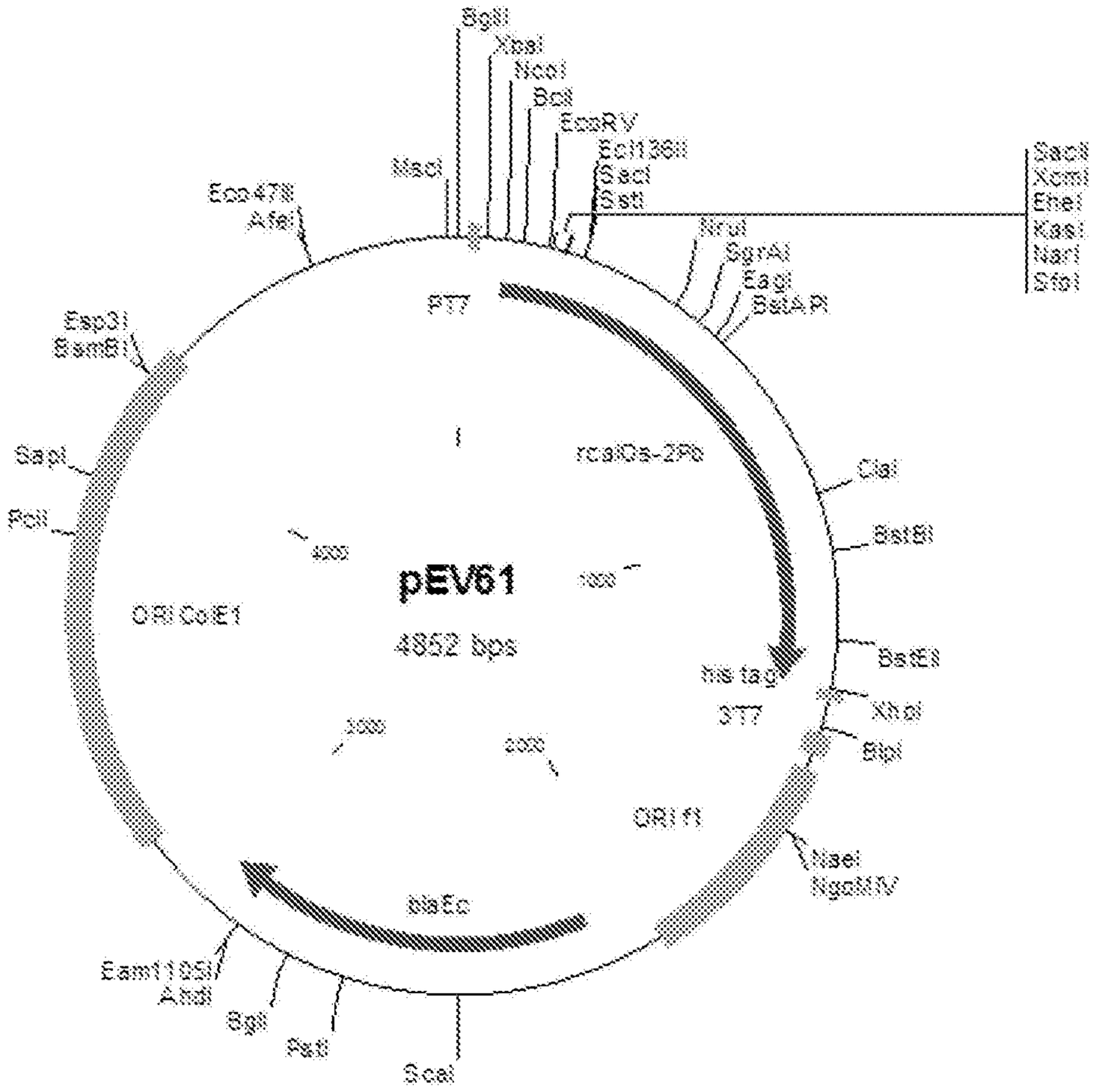

BglII
XbaI
NcoI
ScaI
EcoRV
Ecl136II
SacI
SstI
NruI
SgrAI
PagI
SalA7I
SacI
XcmI
EheI
KasI
NarI
SfoI
NaeI
Eco47III
AfeI
Esp3I
BsmBI
T7
ClaI
BstBI
BstEII
XhoI
BlpI
NheI
NgoMIV
SapI
PciI
ampR
pEV61
4852 bps
rop
ORI ColE1
his tag
3'T7
ORI f1
lacIq
Eam1105I
AflII
BglI
PstI
SceI

Figure 7A

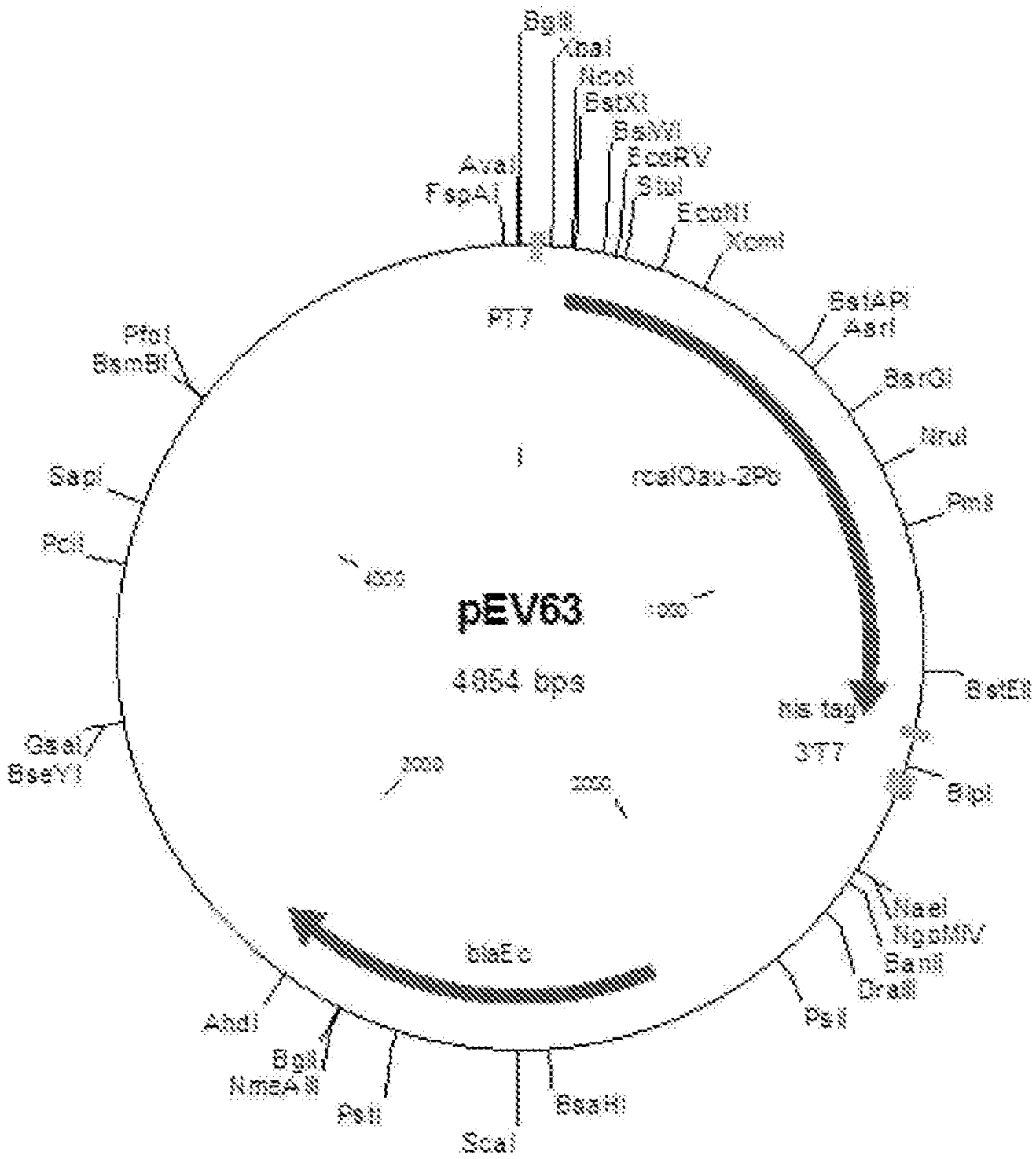

SgfI
XcmI
NcoI
BstXI
BsiWI
EcoRV
SfiI
EcoNI
XcmI
BslAPI
AsiI
BsrGI
NruI
PmlI
BstEII
BlpI
MaeI
NgoMIV
BamHI
DraIII
PsiI
BsaHI
PstI
NmeAIII
BglII
AhdI
SseVI
GsaI
SapI
PciI
PflFI
BsmBI
pEV63
4854 bps
ori
bla
rop
lacI
T7
PT7
his tag
rcaIOau-2P5
ScaI

Figure 7B

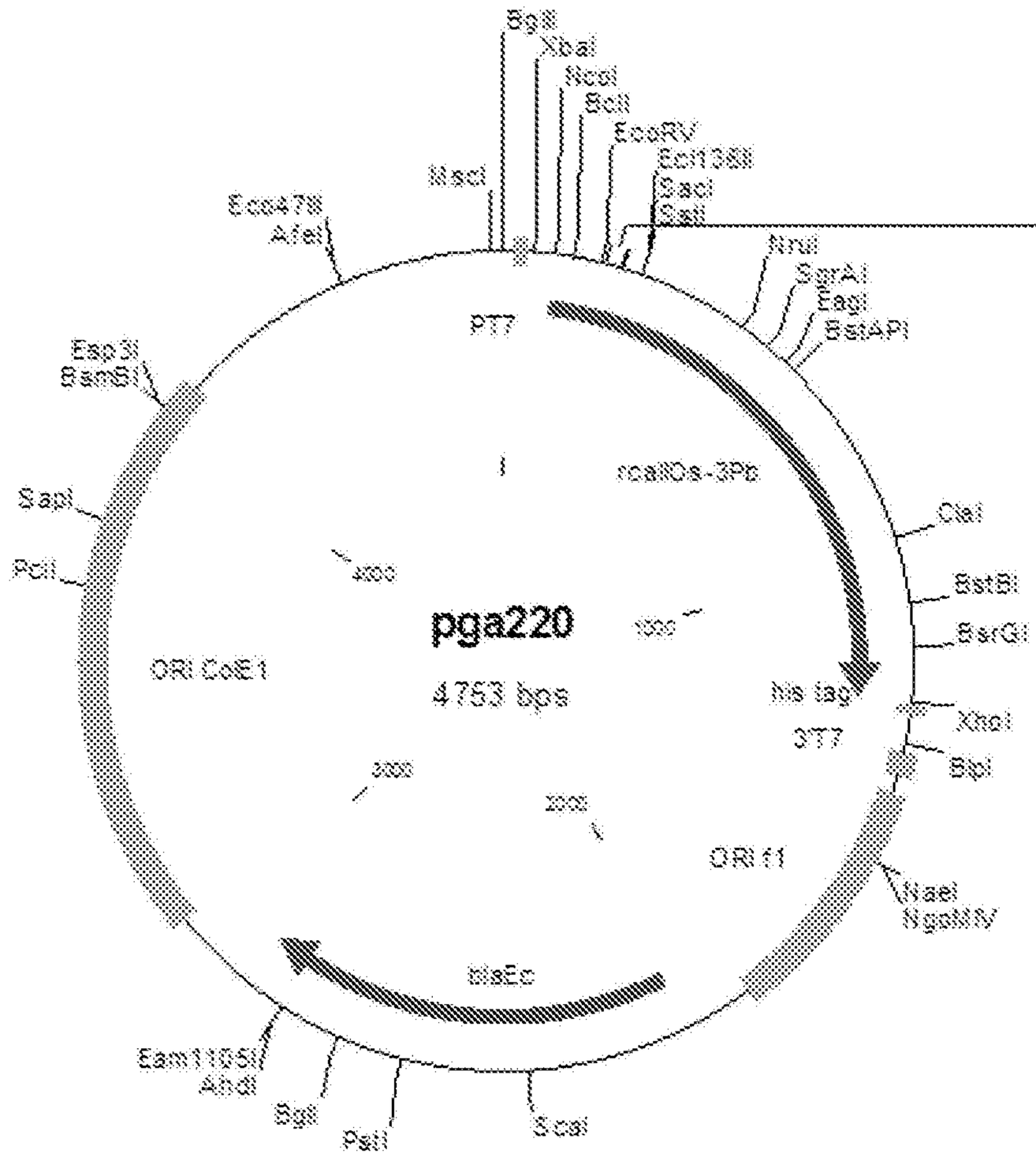

pga220
4753 bps
T7
rcaIOs-3Pb
his tag
3'T7
ORI CoE1
ORI f1
blsEc
Sgfl
XbaI
NcoI
BclI
EcoRV
Ecl136II
SacI
SalI
NruI
SgrAI
EagI
BstAPI
SacII
XcmI
EheI
KasI
NarI
SfoI
ClaI
BstBI
BsrGI
XhoI
BplI
NaeI
NgoMIV
ScaI
PstI
SphI
HindIII
Eam1105I
AhdI
BglII
PciI
SapI
MscI
Eco47III
AfeI
Esp3I
BsmBI

Figure 7C

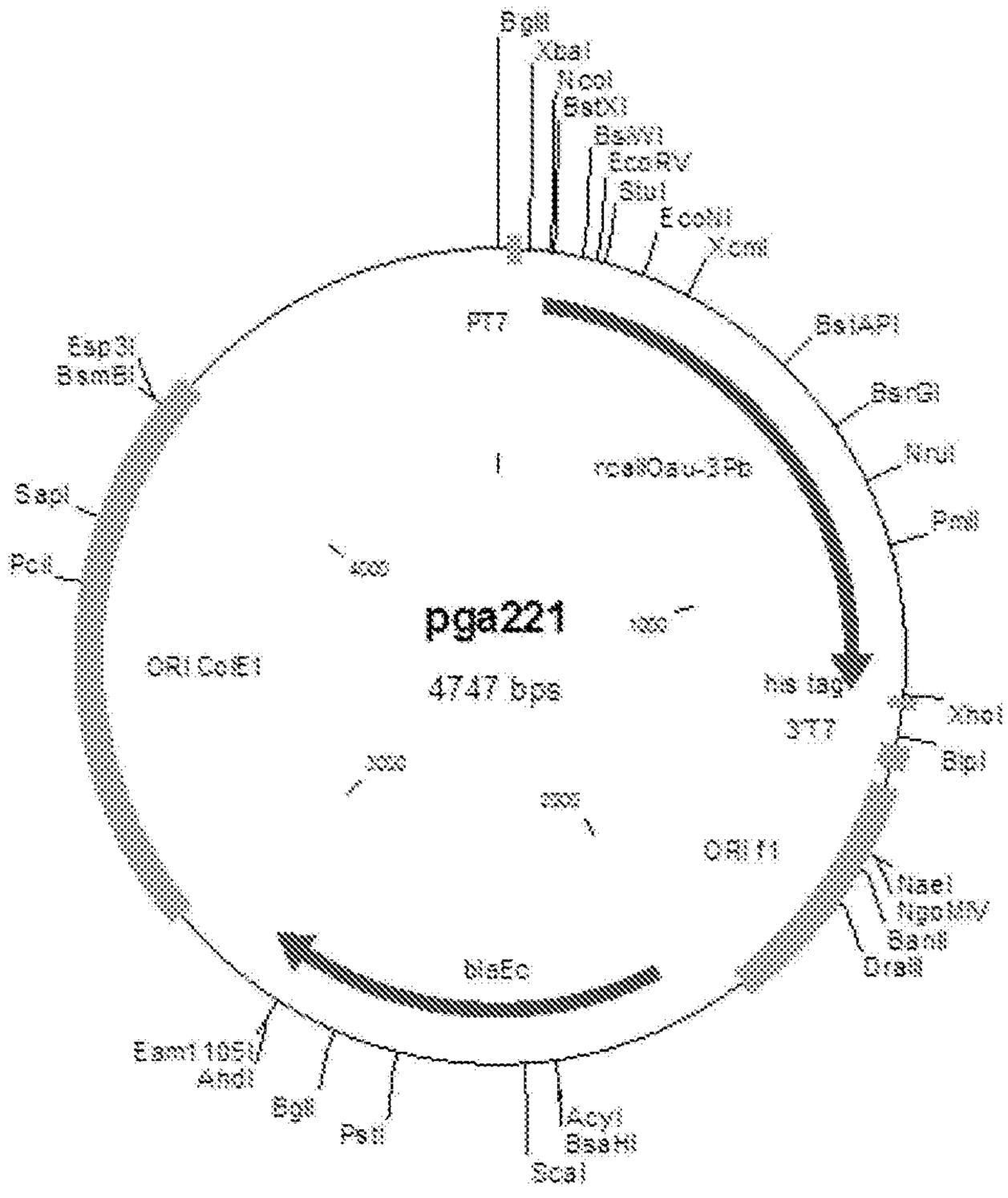

SgfI
XbaI
NcoI
SstI
BglII
EcoRV
StuI
EcoRI
XhoI
PT7
BslAPI
BanGI
NruI
PmlI
rcaIOsa-3Pb
SapI
PciI
xxx
pga221
4747 bps
ORI ColE1
his tag
3'T7
XhoI
SpeI
ORI f1
NaeI
NgoMIV
BamHI
SrfI
NsiEc
EamI105I
AhdI
BglI
PstI
AcyI
BssHI
ScaI

Figure 7D

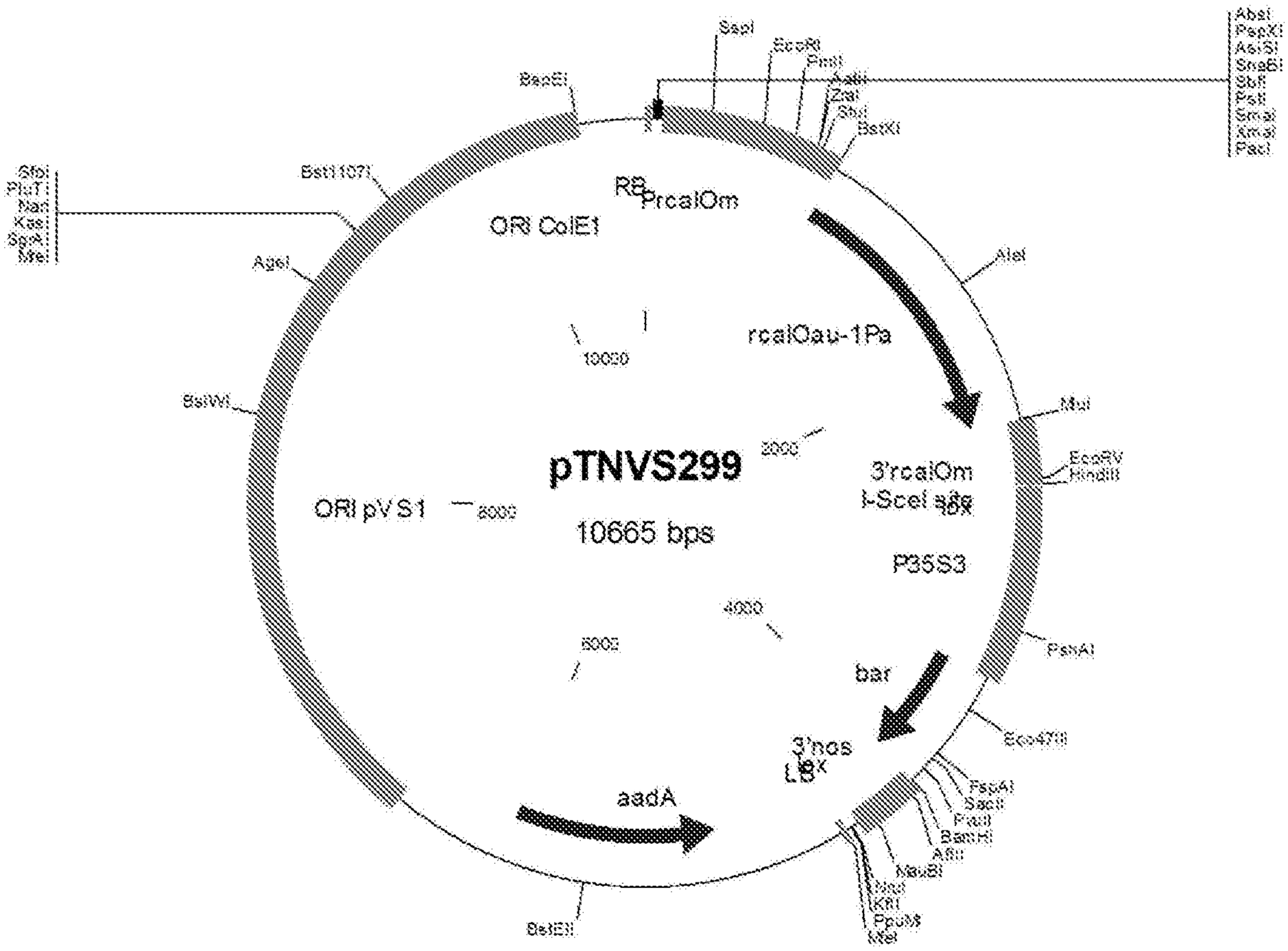

pTNVS299
10665 bps
ORI ColE1
ORI pVS1
RB PrcalOm
rcalOau-1Pa
3'rcalOm
I-SceI site
P35S3
bar
3'nos
LB
aadA
2000
4000
6000
8000
10000
SspI
EcoRI
PmlI
AatII
ZraI
SbfI
BstXI
AleI
MunI
EcoRV
HindIII
PshAI
Eco47III
FspAI
SacII
PvuII
BamHI
AflII
MauBI
NruI
KpnI
PpuMI
NheI
BstEII
BsiWI
AgeI
Bst1107I
SfoI
PluTI
NarI
KasI
SgrAI
MfeI
BspEI
AscI
PspXI
AsiSI
SnaBI
SbfI
PstI
SmaI
XmaI
PacI

Figure 7E

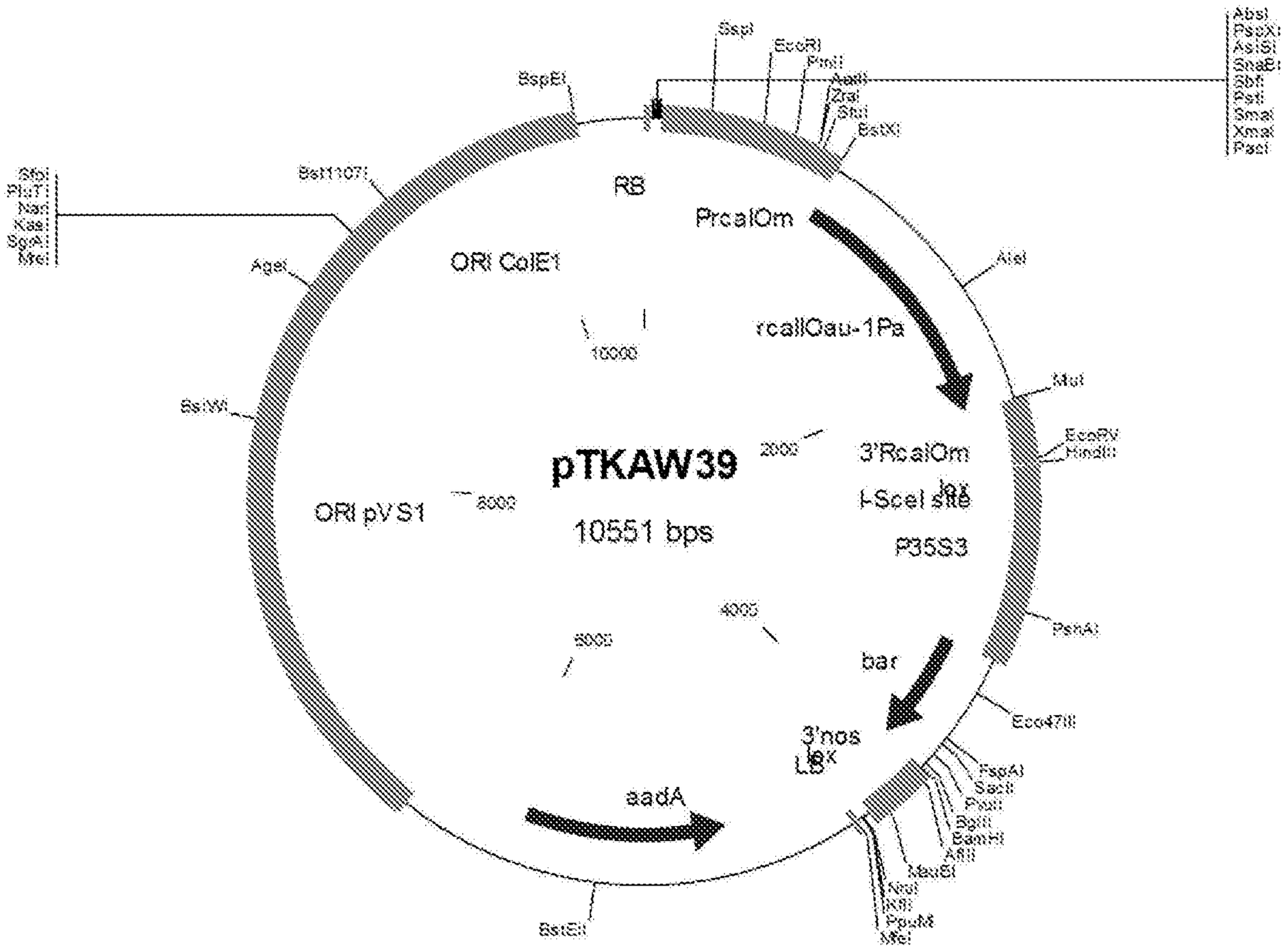

pTKAW39
10551 bps
RB
PrcalOm
rcallOau-1Pa
3'RcalOm
I-SceI site
P35S3
bar
3'nos
LB
aadA
ORI ColE1
ORI pVS1
2000
4000
6000
8000
10000
AbsI
PspXI
AsiSI
SnaBI
SbfI
SstI
SmaI
XmaI
PacI
EcoRI
PmlI
AatII
ZraI
SfuI
BstXI
BspEI
Bst1107I
SfoI
PluTI
NaeI
KasI
SgrAI
MfeI
AgeI
BsiWI
AleI
MluI
EcoRV
HindIII
PshAI
Eco47III
FspAI
SacII
PstI
BglII
BamHI
AflII
MauBI
NruI
KflI
PpuMI
MfeI
BstEII

Figure 7F

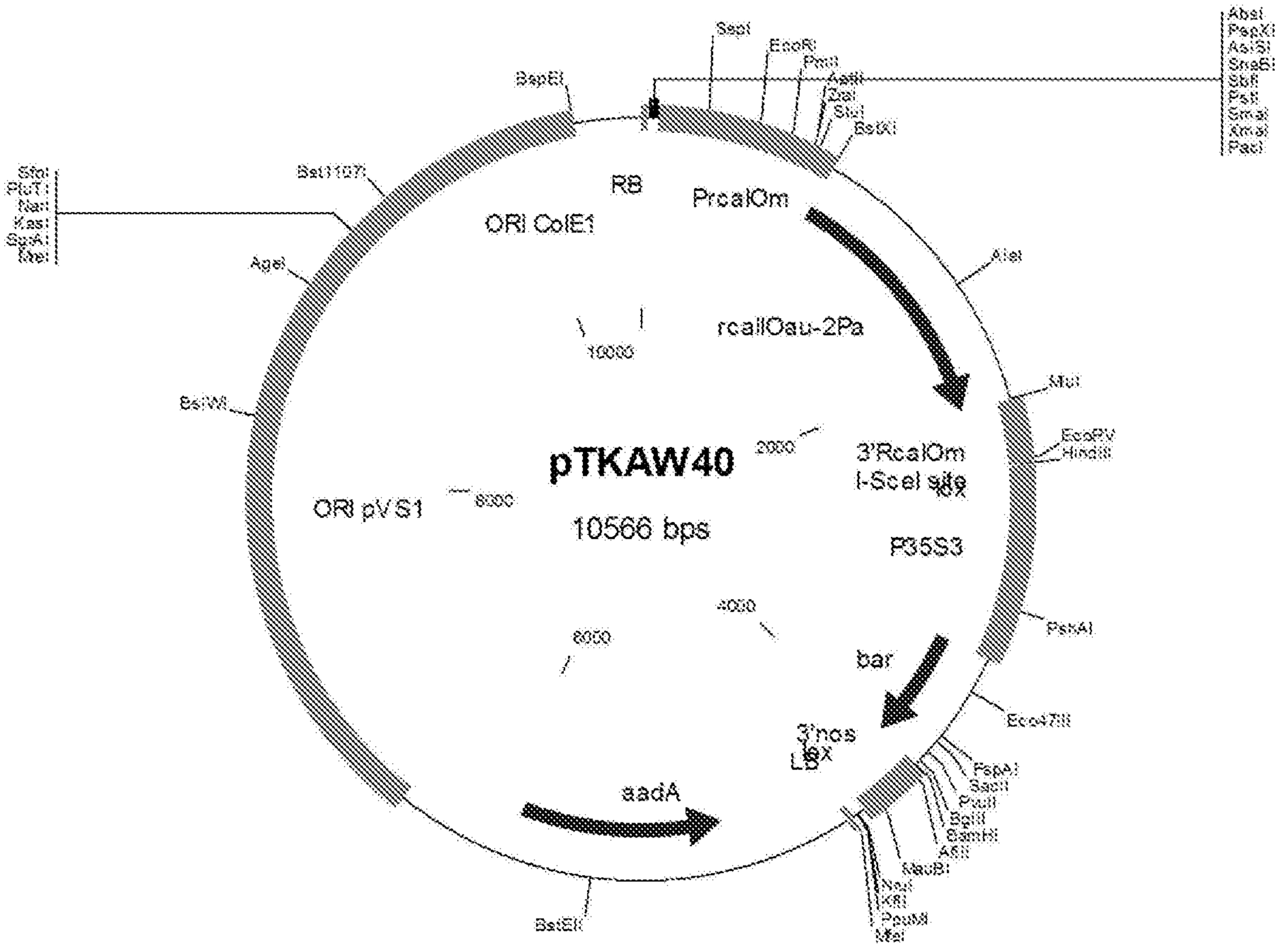

RB
PrcalOm
ORI ColE1
rcallOau-2Pa
pTKAW40
10566 bps
3'RcalOm
I-SceI site
P35S3
ORI pVS1
bar
3'nos
LB
aadA
10000
2000
4000
6000
8000
SspI
EcoRI
PmlI
AseRI
ClaI
StuI
BstXI
BspEI
Bst1107I
SfoI
PluTI
NarI
KasI
SgrAI
XbaI
AgeI
AleI
BsiWI
MluI
EcoRV
HindIII
PshAI
Eco47III
FspAI
SacII
PvuII
BglII
BamHI
AflII
NaeBI
NsiI
XhoI
PpuMI
NheI
BstEII
AbaI
PspXI
AscSI
SnaBI
SbfI
PstI
SmaI
XmaI
PacI

Figure 7G

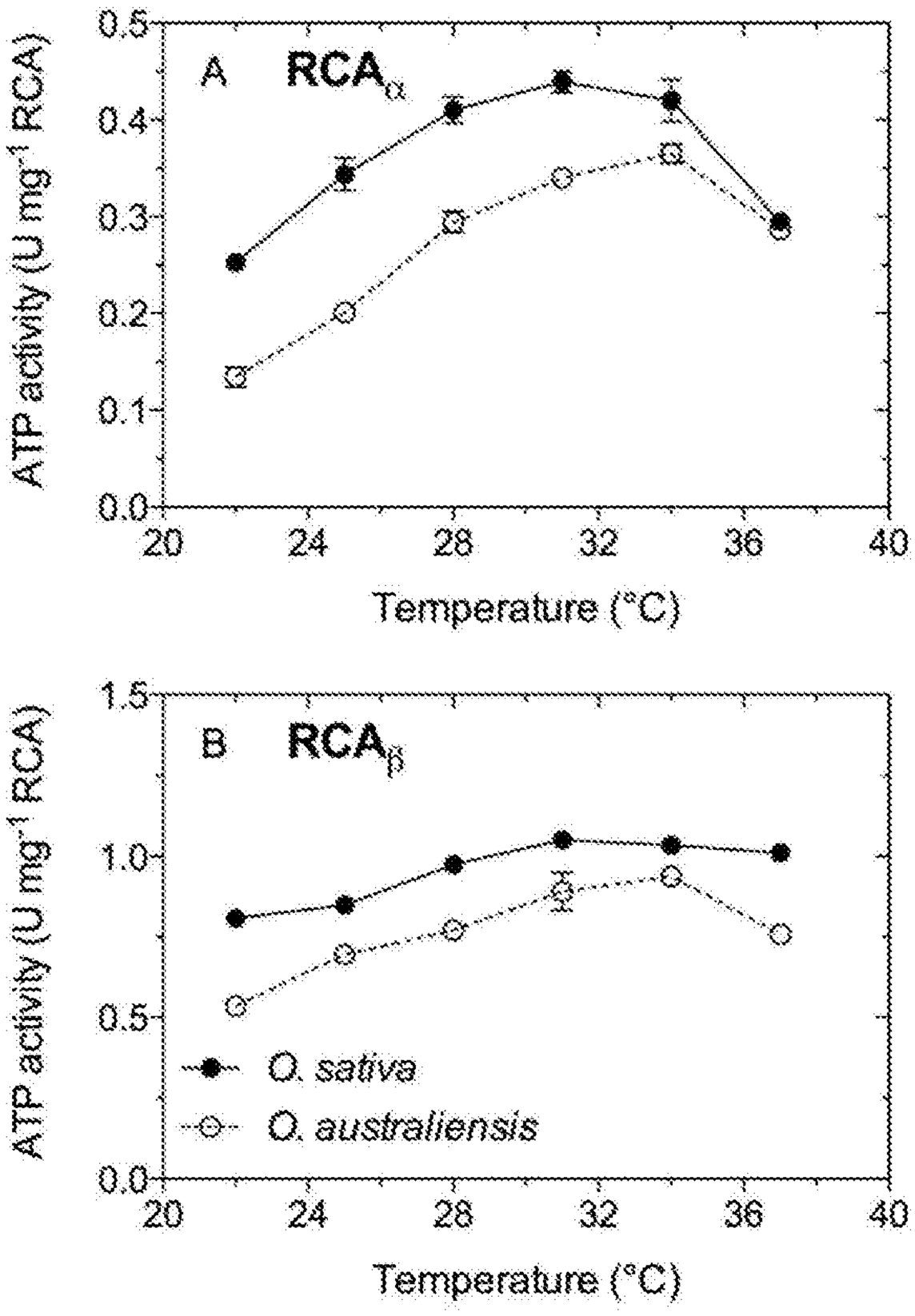

A
RCA$_\alpha$
ATP activity (U mg^{-1} RCA)
0.0
0.1
0.2
0.3
0.4
0.5
20
24
28
32
36
40
Temperature (°C)
B
RCA$_\beta$
ATP activity (U mg^{-1} RCA)
0.0
0.5
1.0
1.5
O. sativa
O. australiensis
20
24
28
32
36
40
Temperature (°C)

Figure 8

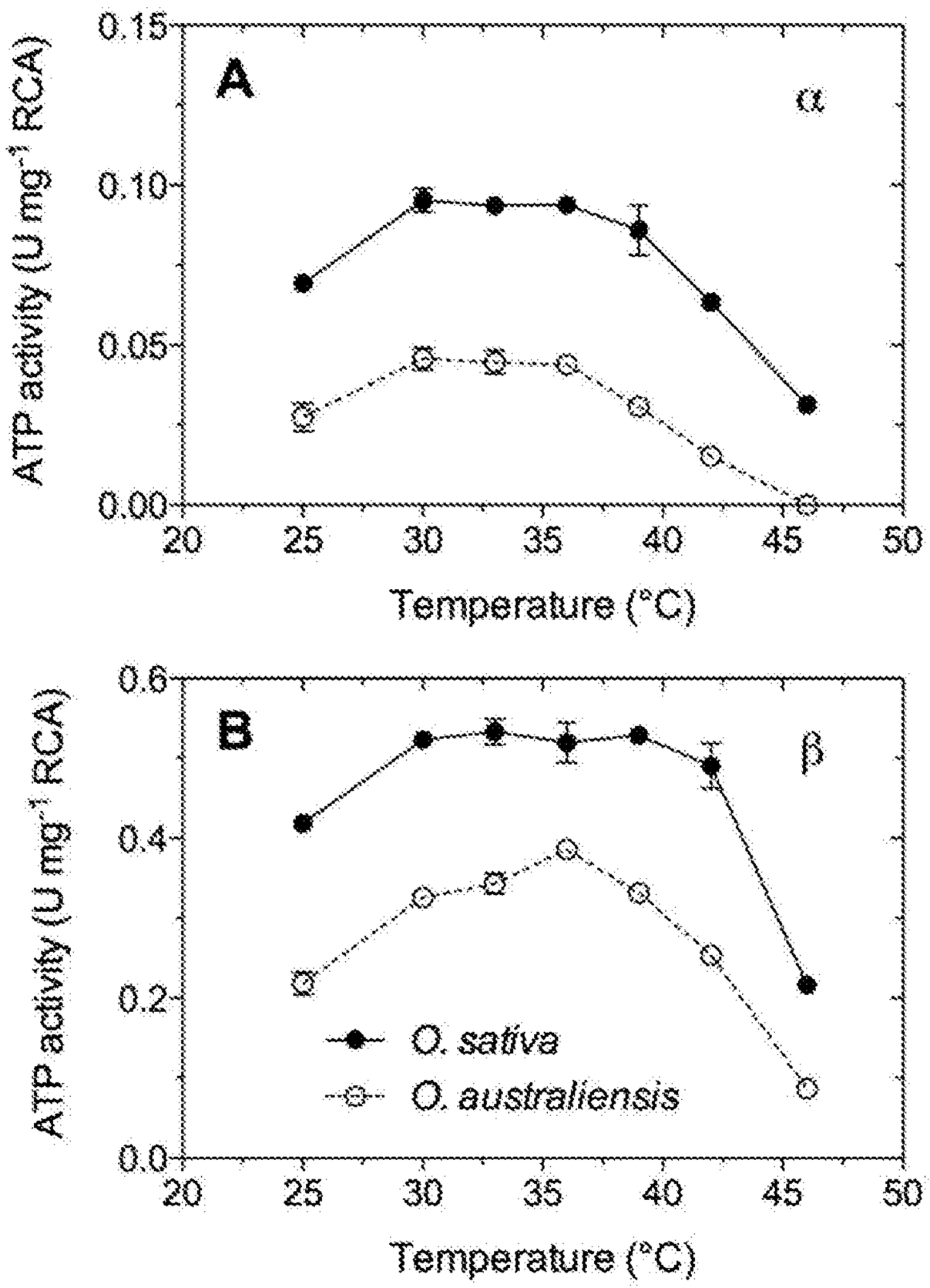

A
α
ATP activity (U mg-1 RCA)
0.15
0.10
0.05
0.00
20
25
30
35
40
45
50
Temperature (°C)
B
β
ATP activity (U mg-1 RCA)
0.6
0.4
0.2
0.0
O. sativa
O. australiensis
20
25
30
35
40
45
50
Temperature (°C)

Figure 9

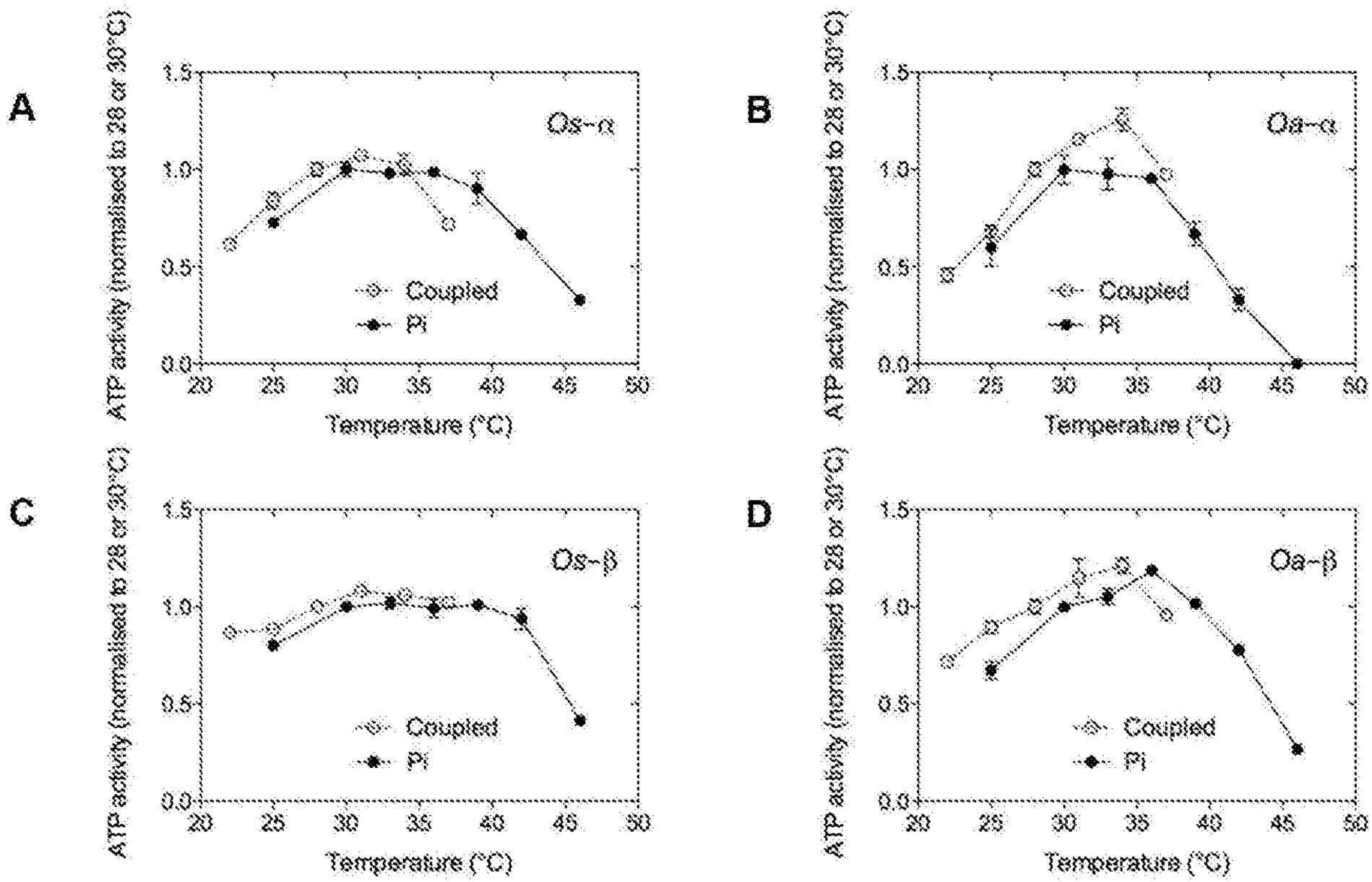

A
B
C
D
Os-α
Oa-α
Os-β
Oa-β
Coupled
Pi
ATP activity (normalised to 28 or 30°C)
Temperature (°C)
0.0
0.5
1.0
1.5
20
25
30
35
40
45
50

Figure 10

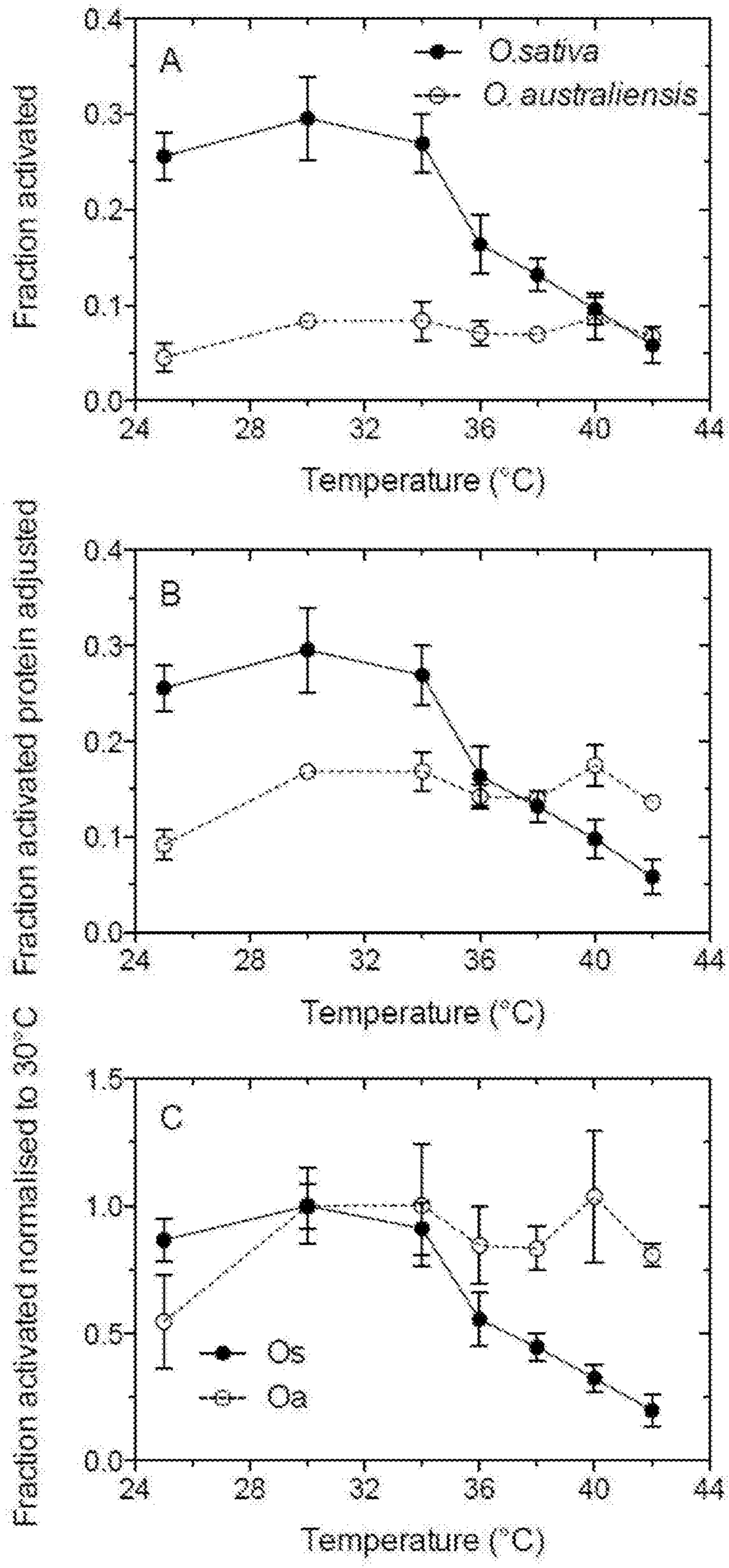

A
O.sativa
O. australiensis
Fraction activated
0.4
0.3
0.2
0.1
0.0
24
28
32
36
40
44
Temperature (°C)
B
Fraction activated protein adjusted
C
Os
Oa
Fraction activated normalised to 30°C
1.5
1.0
0.5

Figure 11

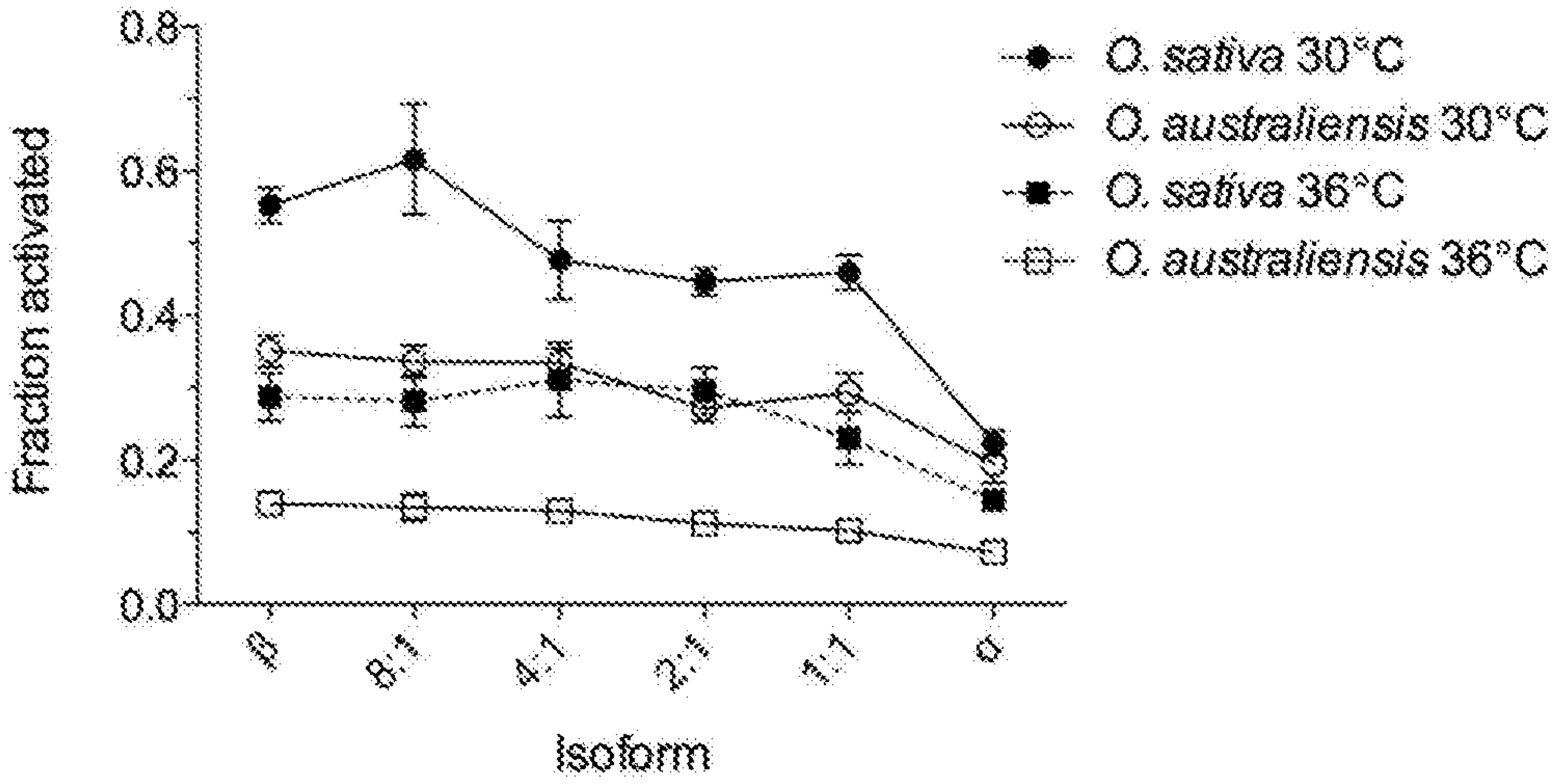


Figure 12

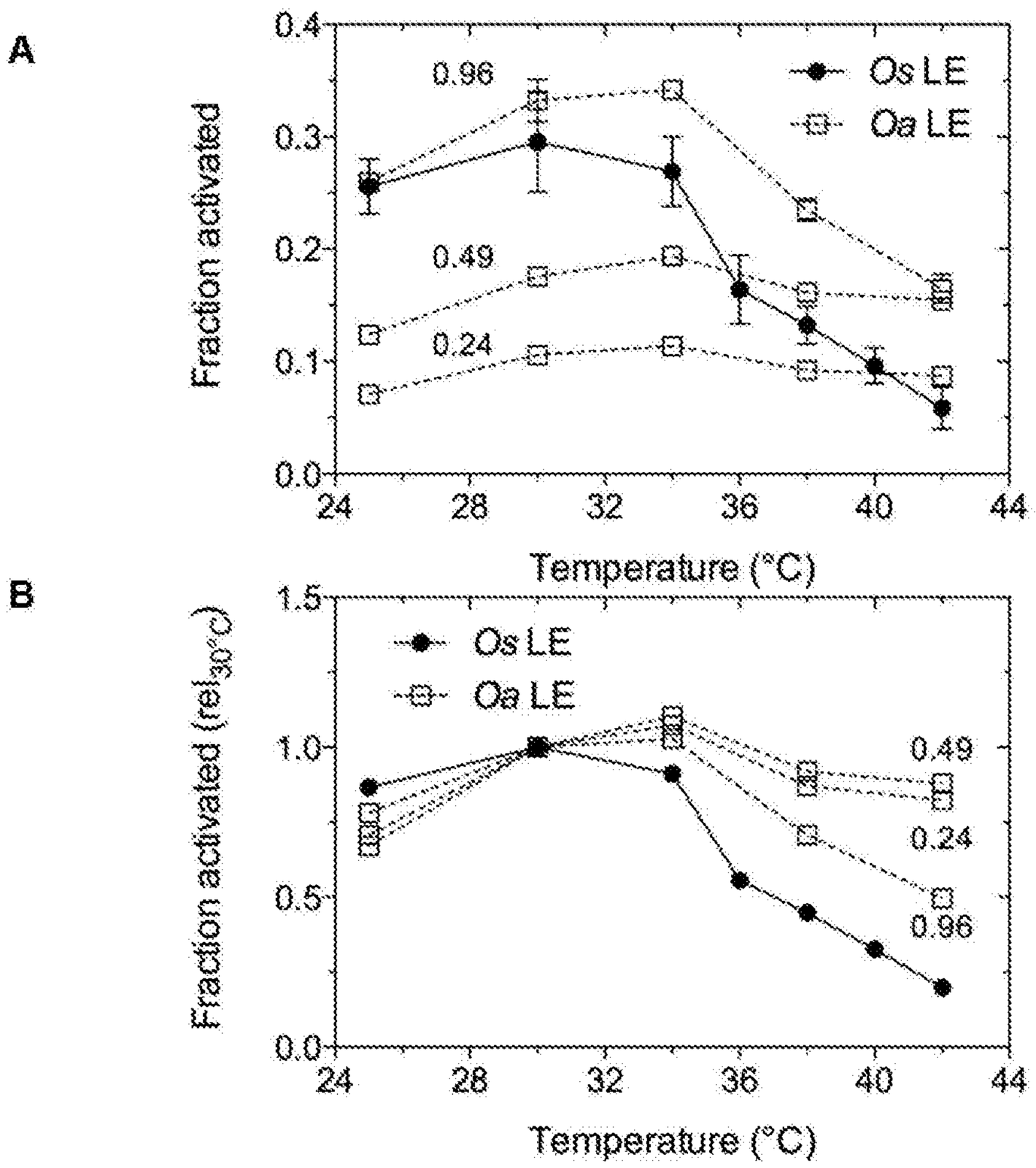


Figure 13

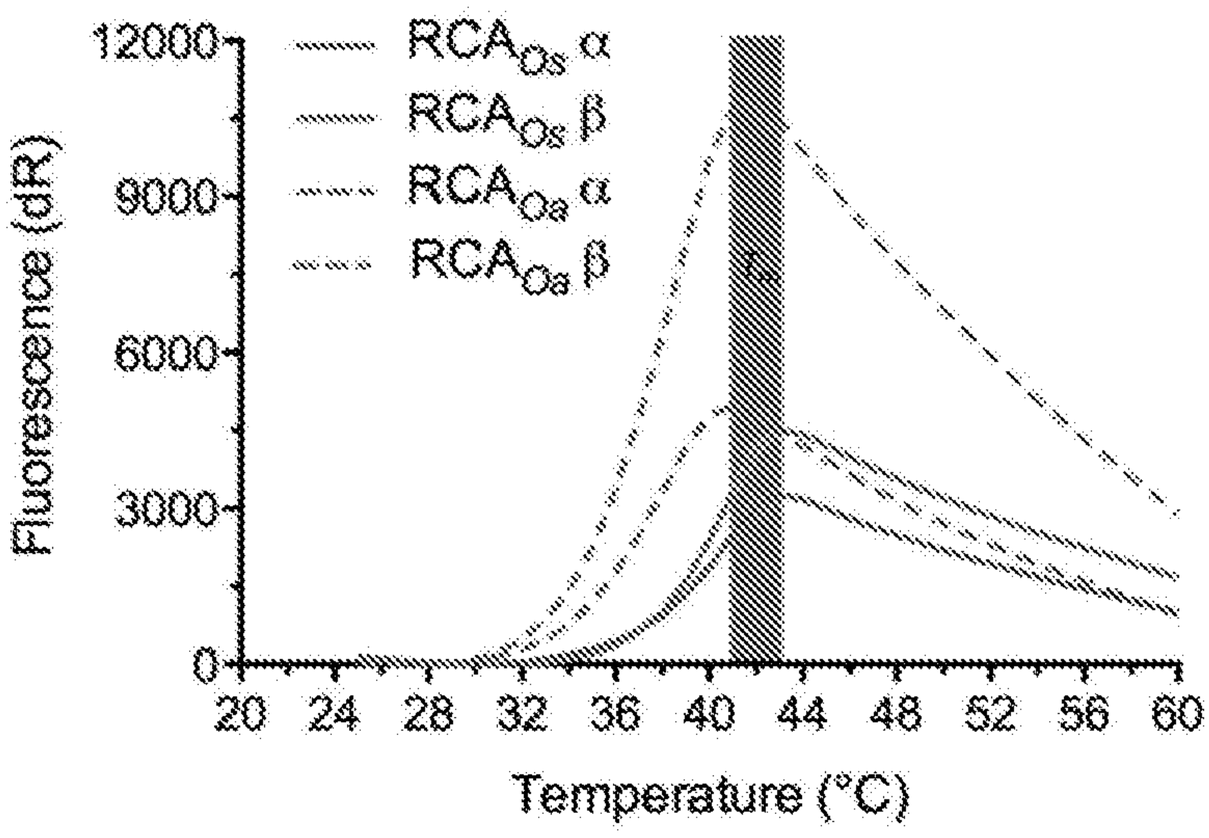


Figure 14

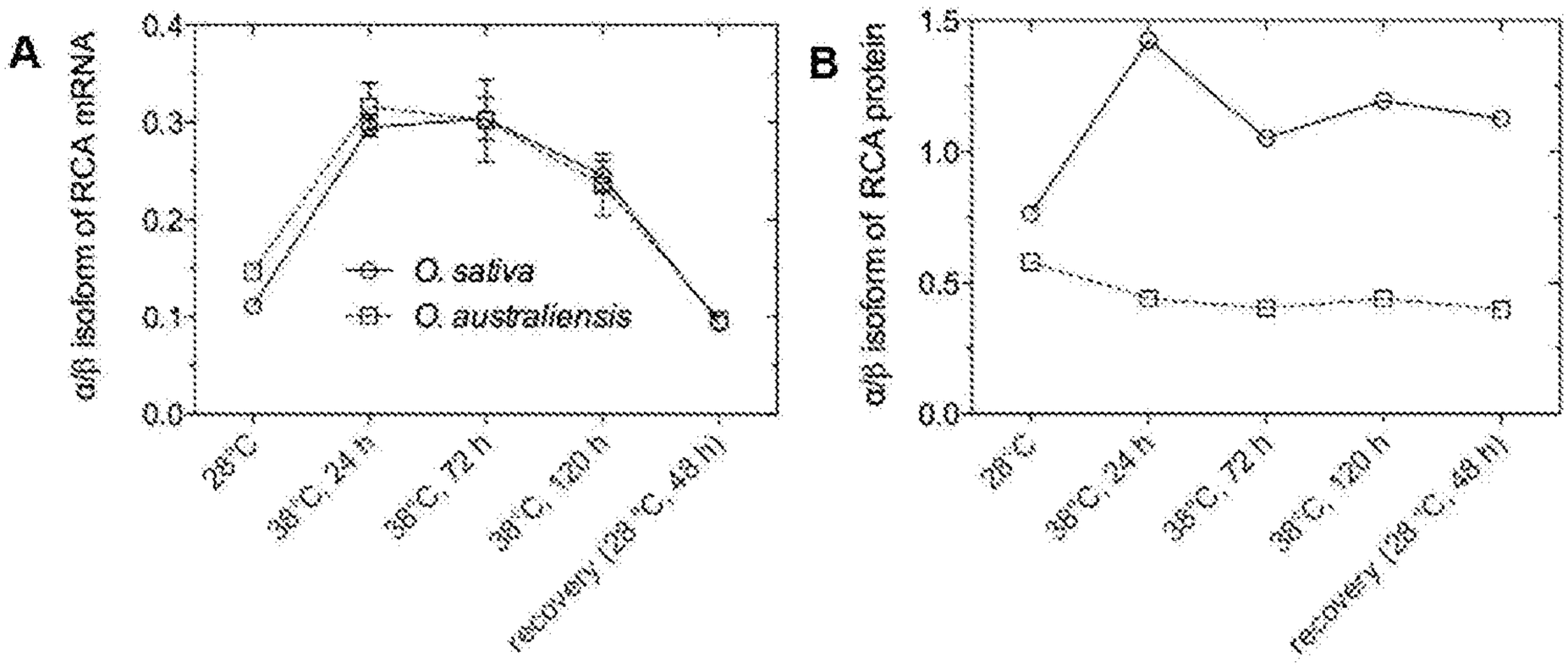
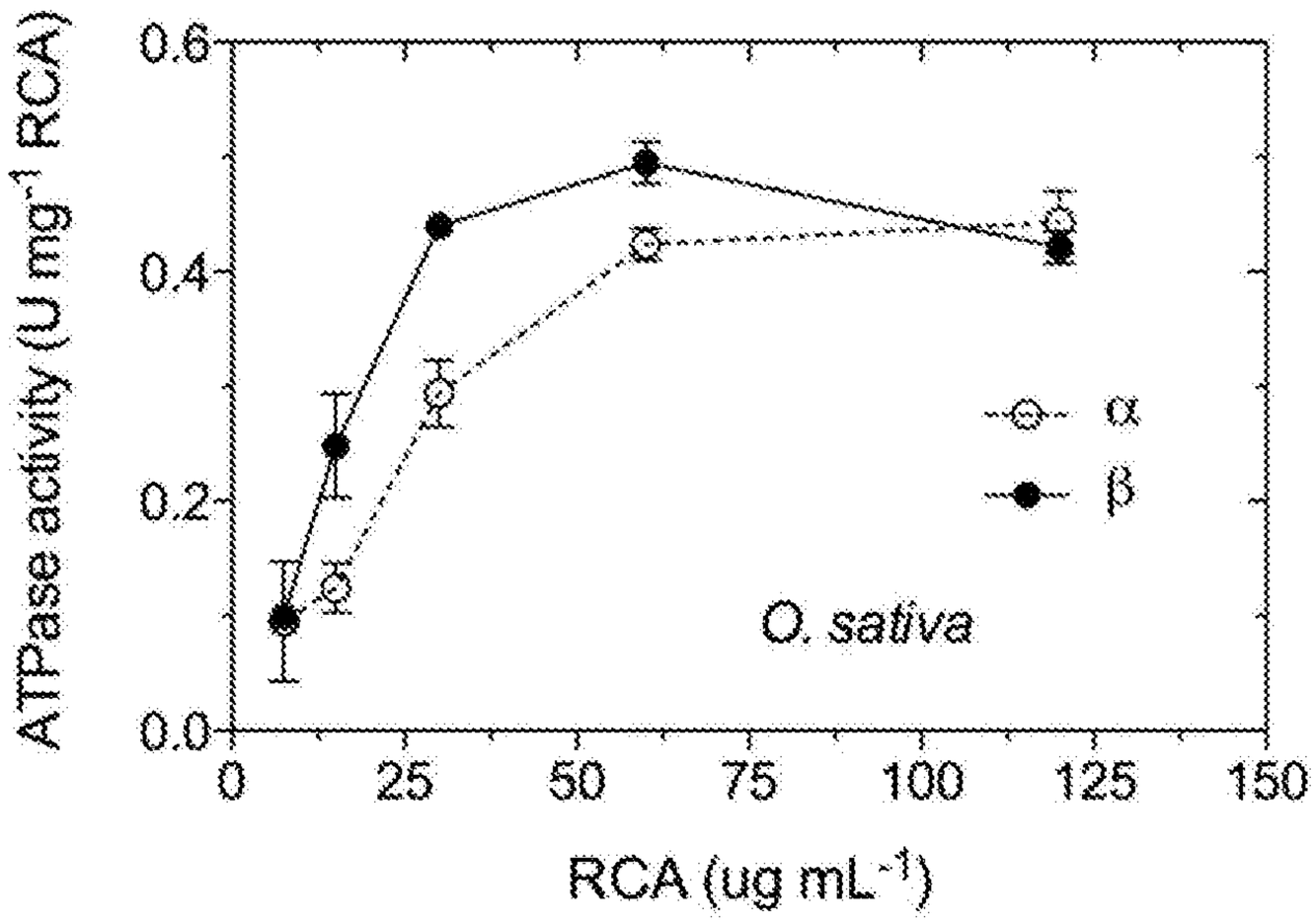


Figure 15

A


ATPase activity (U mg-1 RCA)
0.6
0.4
0.2
0.0
0
25
50
75
100
125
150
RCA (ug mL-1)
α
β
O. sativa

B

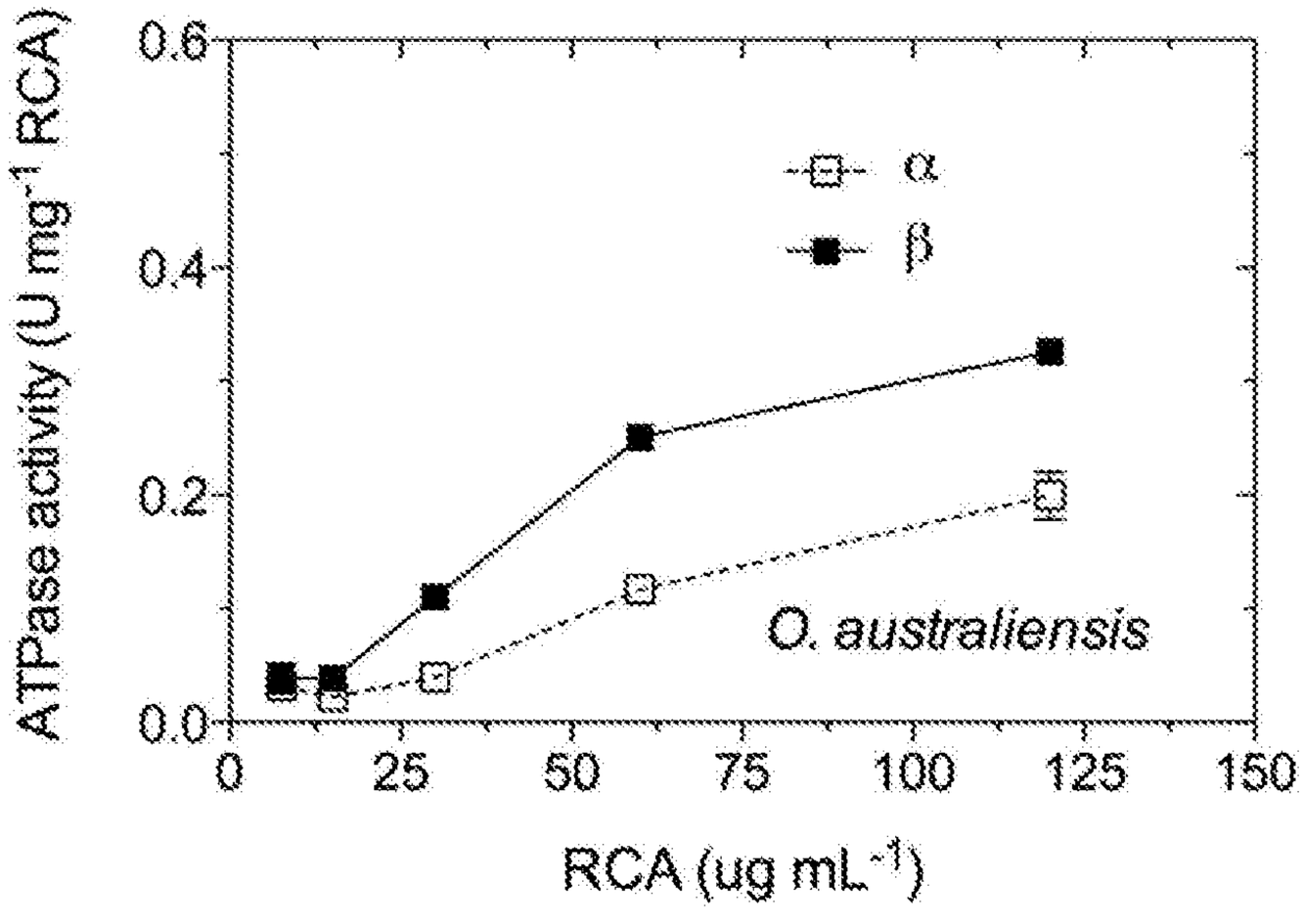

ATPase activity (U mg-1 RCA)
0.6
0.4
0.2
0.0
0
25
50
75
100
125
150
RCA (ug mL-1)
α
β
O. australiensis

Figure 16

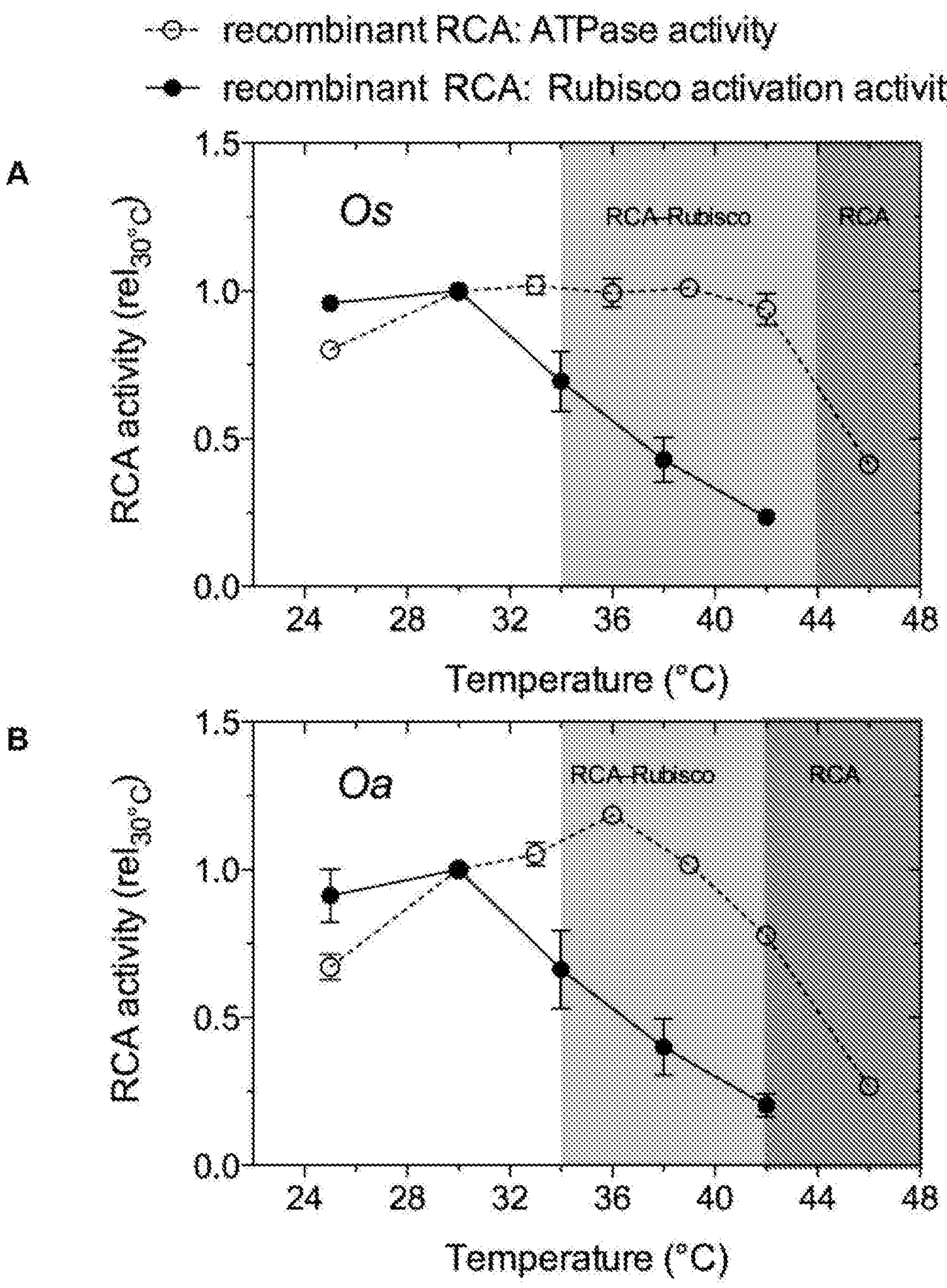

recombinant RCA: ATPase activity
recombinant RCA: Rubisco activation activity
A
Os
RCA-Rubisco
RCA
RCA activity (rel30°C)
1.5
1.0
0.5
0.0
24
28
32
36
40
44
48
Temperature (°C)
B
Oa
RCA-Rubisco
RCA
RCA activity (rel30°C)
1.5
1.0
0.5
0.0
24
28
32
36
40
44
48
Temperature (°C)

Figure 17

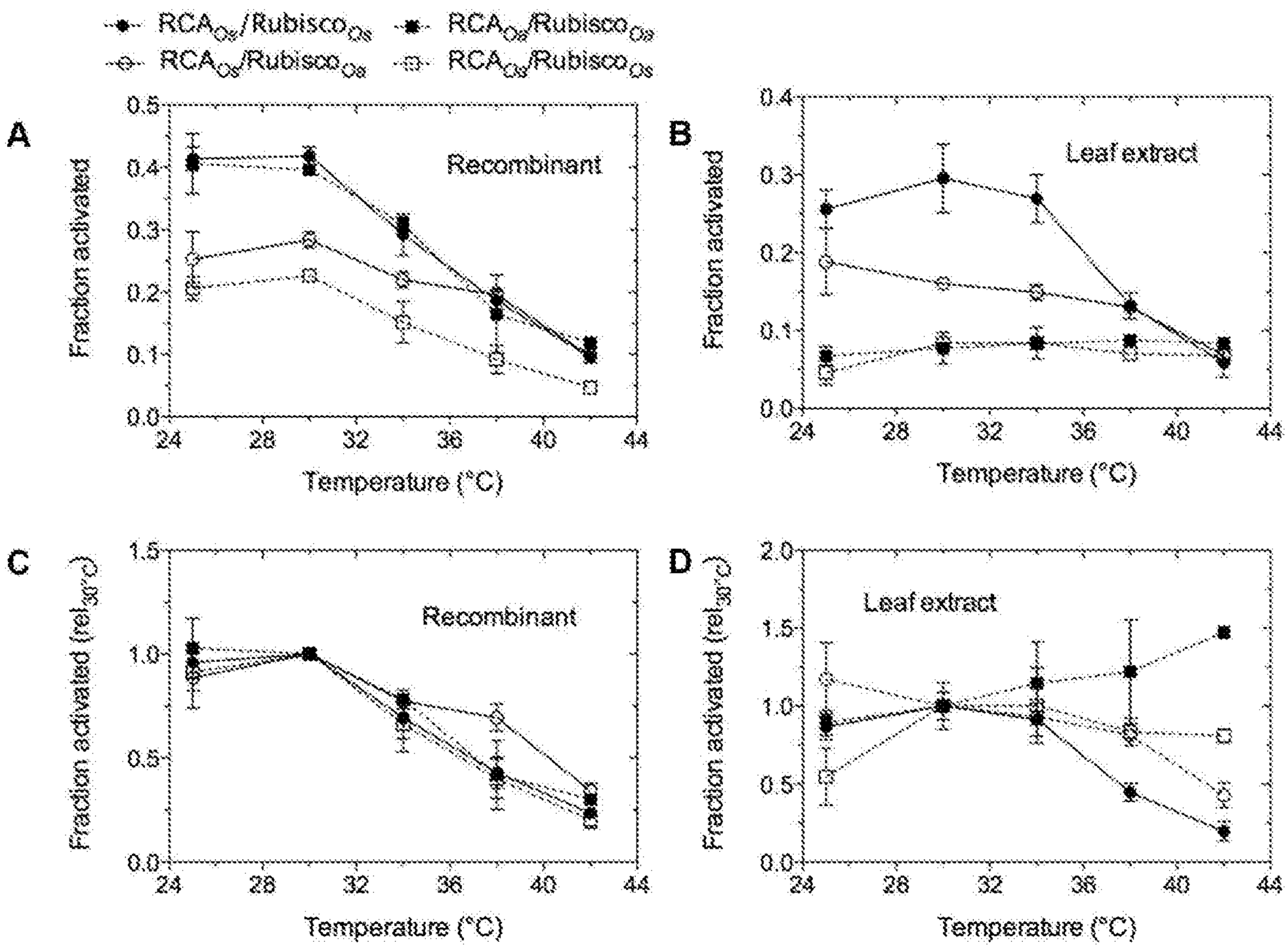

RCA$_{Os}$/Rubisco$_{Os}$
RCA$_{Os}$/Rubisco$_{Os}$
RCA$_{Os}$/Rubisco$_{Os}$
RCA$_{Os}$/Rubisco$_{Os}$
A
Recombinant
Fraction activated
Temperature (°C)
B
Leaf extract
Fraction activated
Temperature (°C)
C
Recombinant
Fraction activated (rel$_{30°C}$)
Temperature (°C)
D
Leaf extract
Fraction activated (rel$_{30°C}$)
Temperature (°C)

Figure 18

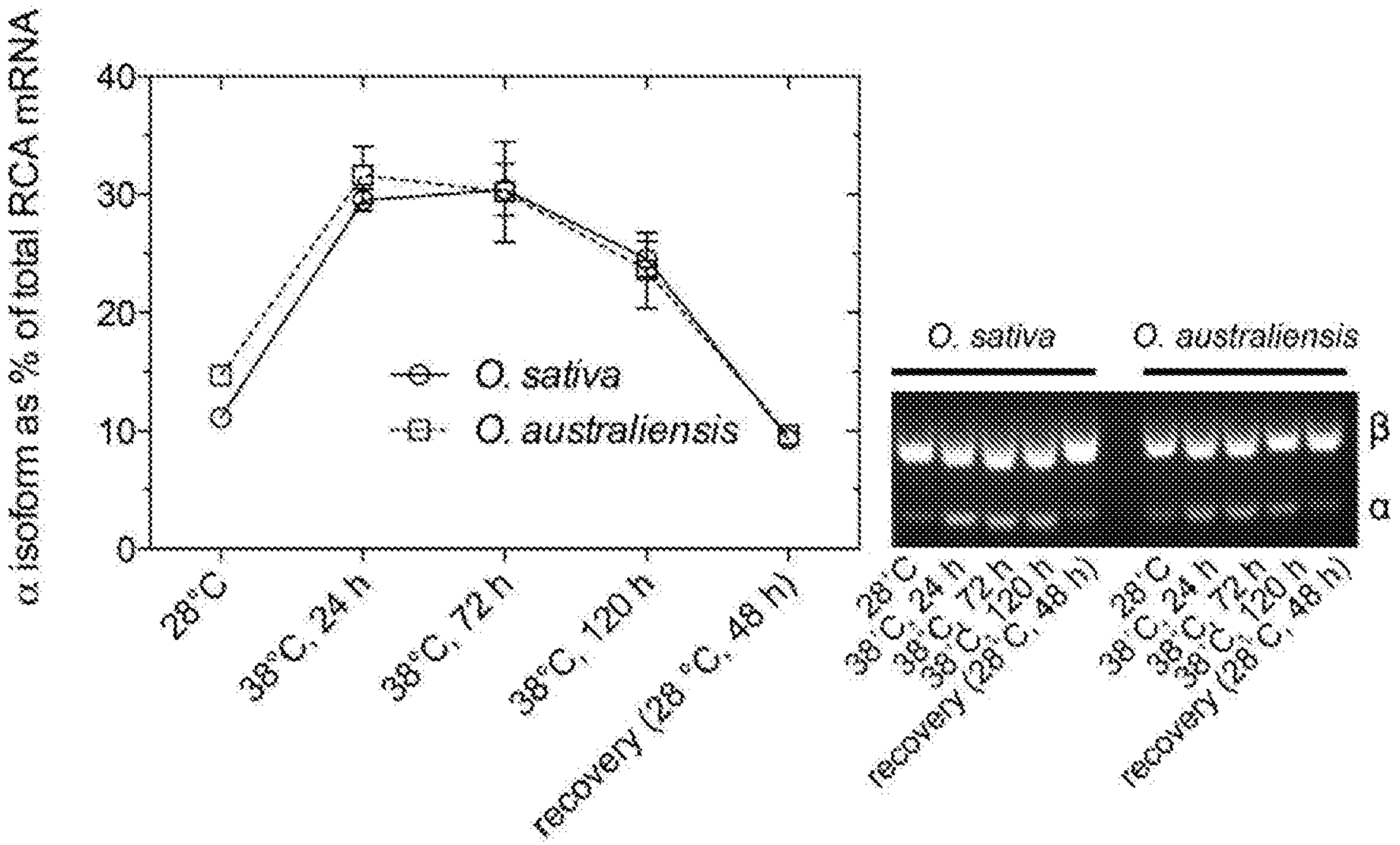


Figure 19

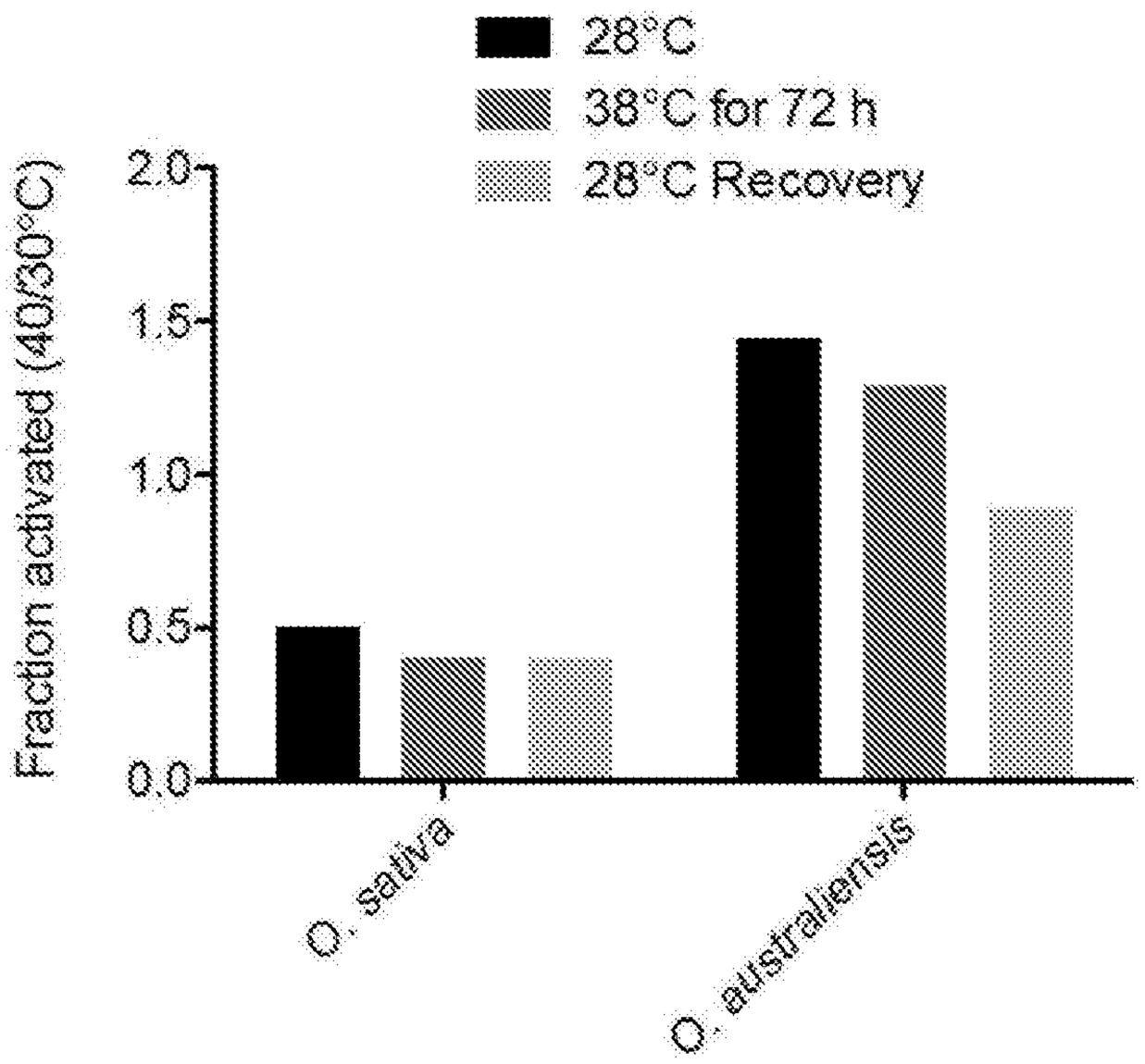


Figure 20

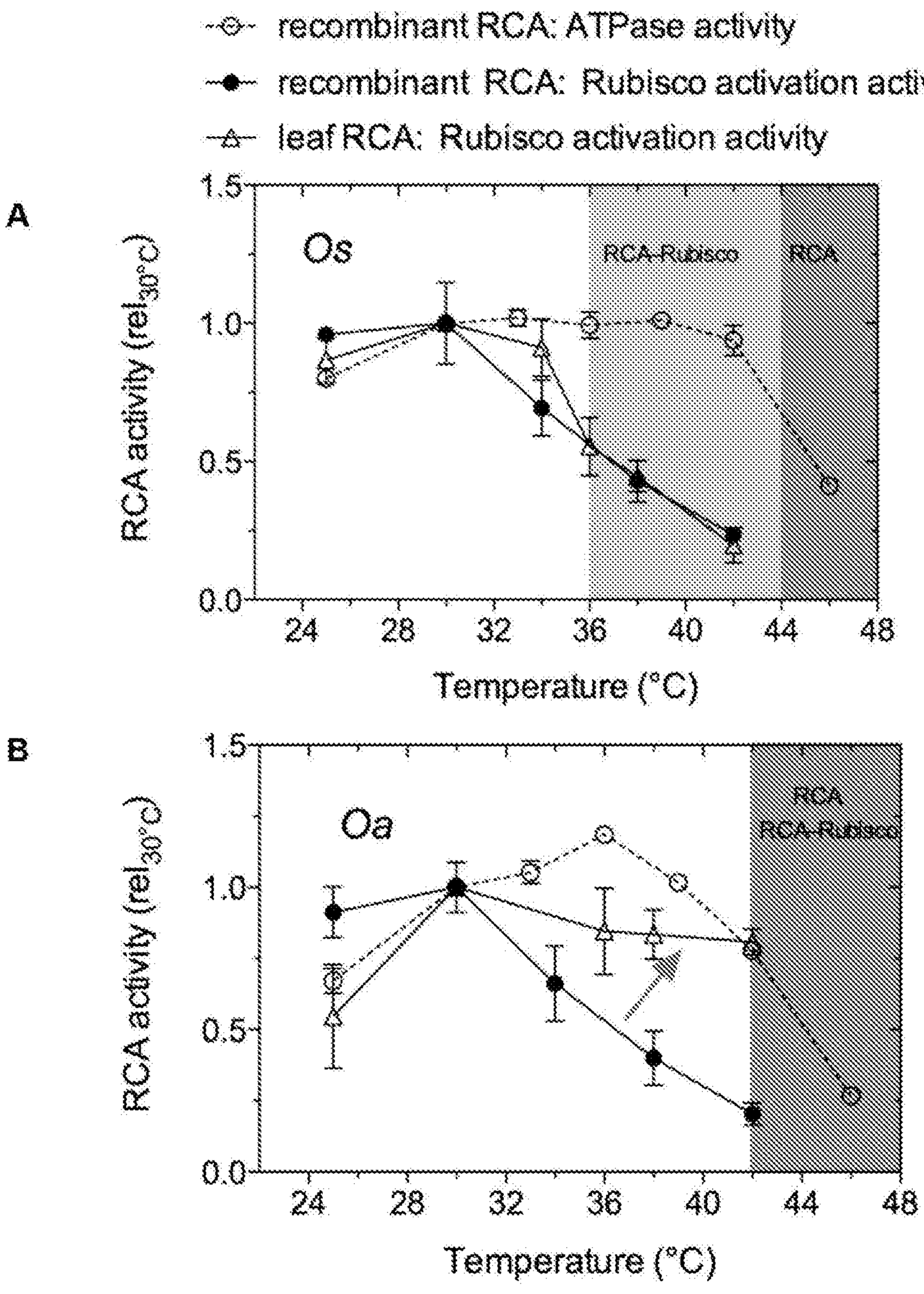

recombinant RCA: ATPase activity
recombinant RCA: Rubisco activation activity
leaf RCA: Rubisco activation activity
A
Os
RCA-Rubisco
RCA
RCA activity (rel30°C)
1.5
1.0
0.5
0.0
24
28
32
36
40
44
48
Temperature (°C)
B
Oa
RCA
RCA-Rubisco
RCA activity (rel30°C)
1.5
1.0
0.5
0.0
24
28
32
36
40
44
48
Temperature (°C)

Figure 21

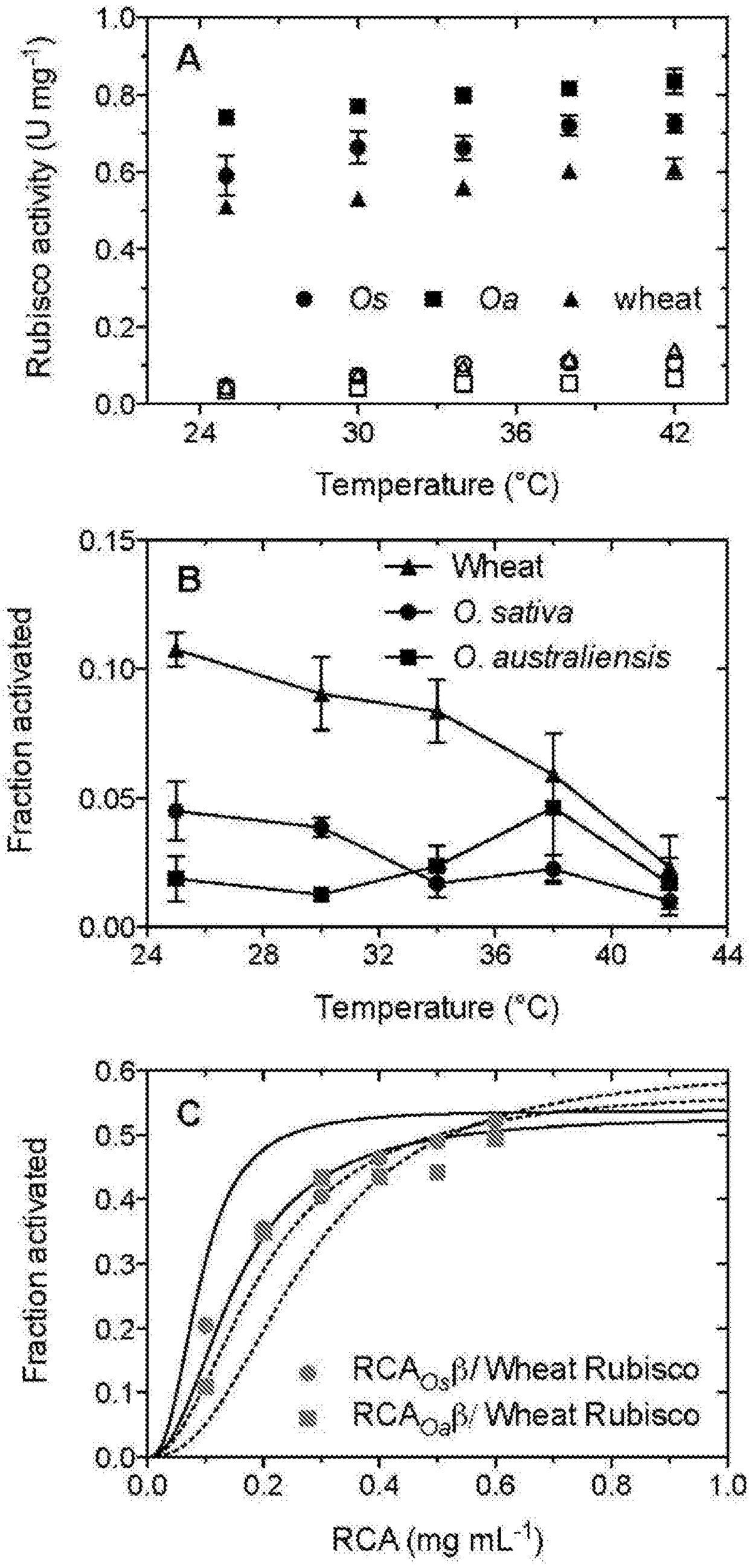

A
Rubisco activity (U mg-1)
1.0
0.8
0.6
0.4
0.2
0.0
Os
Oa
wheat
24
30
36
42
Temperature (°C)
B
Fraction activated
0.15
0.10
0.05
0.00
Wheat
O. sativa
O. australiensis
24
28
32
36
40
44
Temperature (°C)
C
Fraction activated
0.6
0.5
0.4
0.3
0.2
0.1
0.0
RCAOsβ/ Wheat Rubisco
RCAOaβ/ Wheat Rubisco
0.0
0.2
0.4
0.6
0.8
1.0
RCA (mg mL-1)

Figure 22

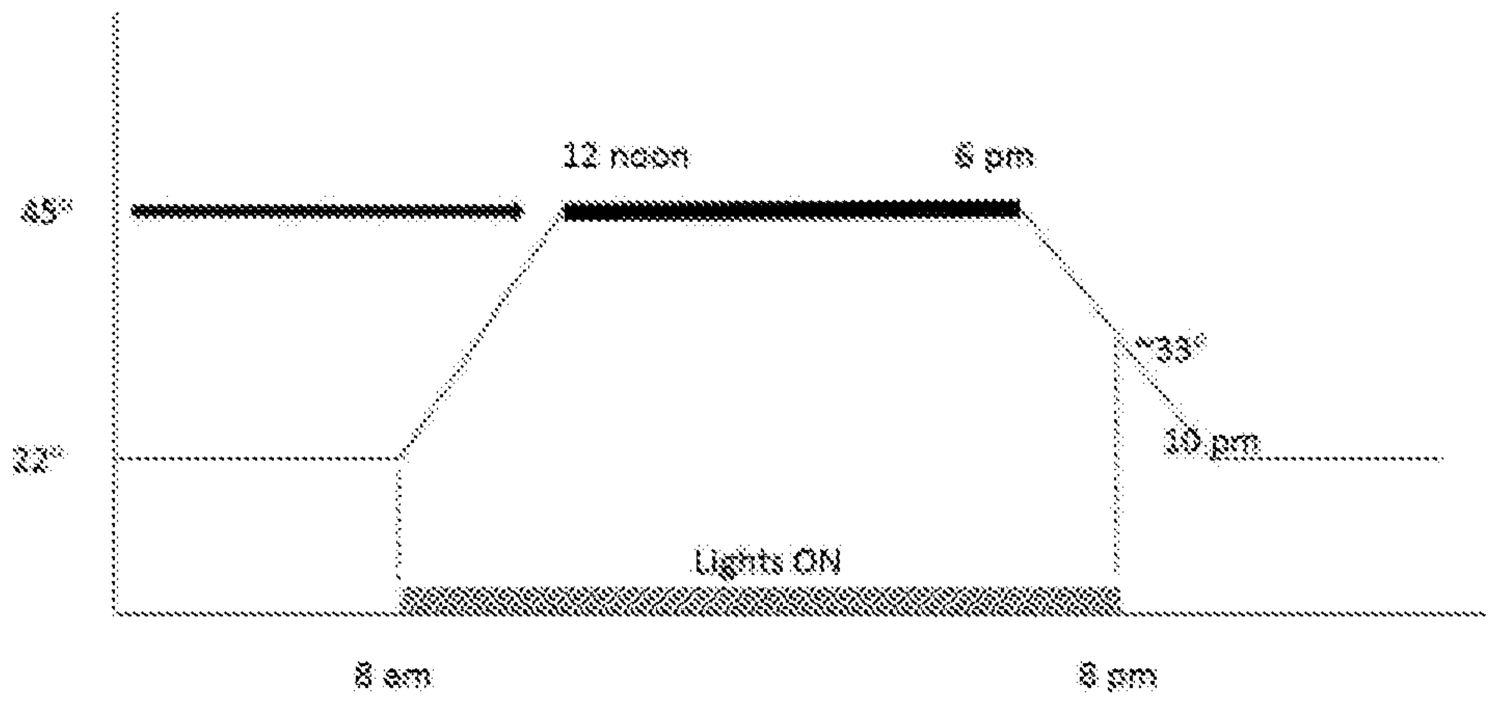
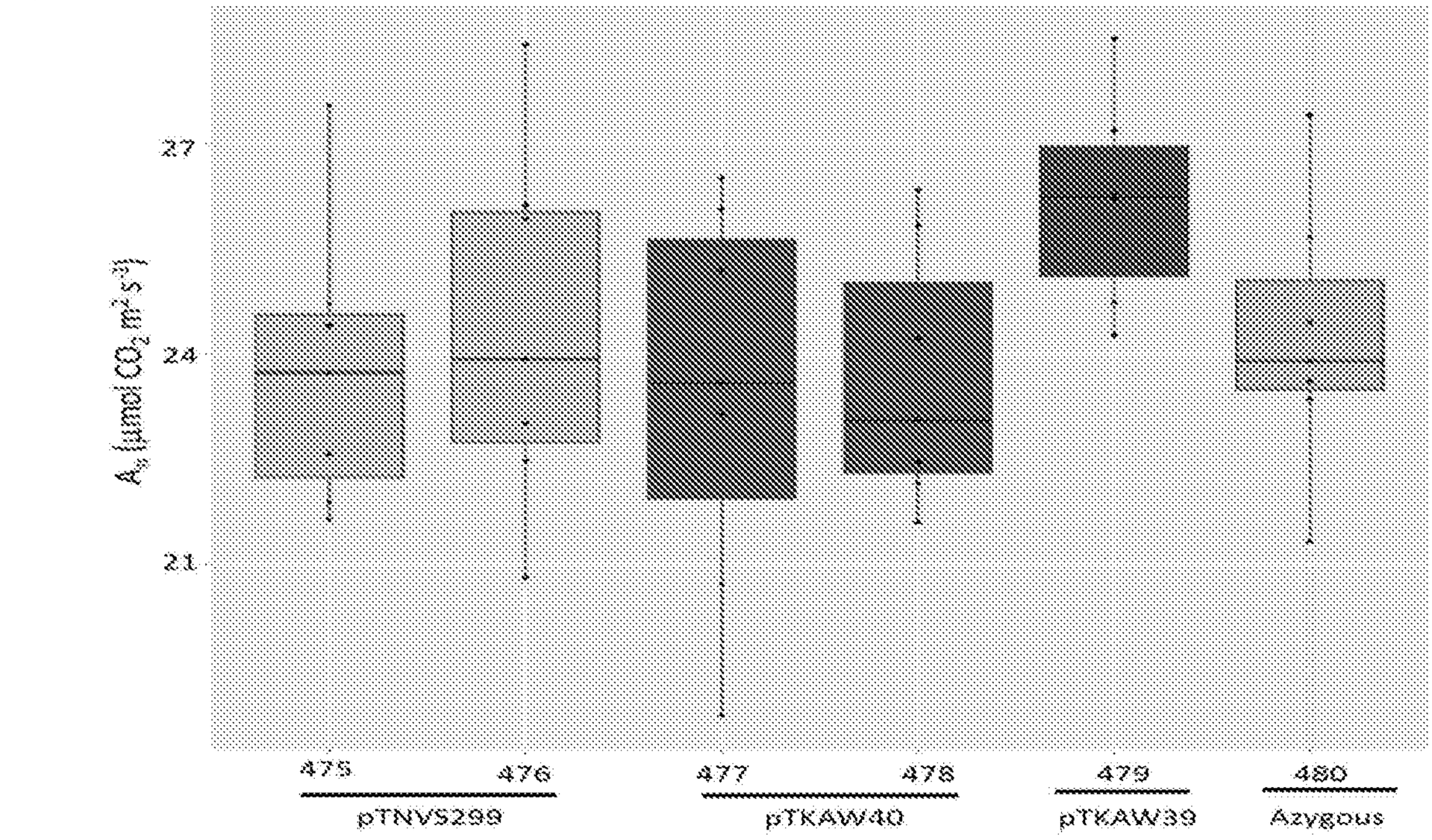

Temperature & light regime – Room 29 (Atwell): 15GTO008
12 noon
6 pm
~33°
10 pm
Lights ON
8 am
6 pm

Figure 23


A
27
24
21
A_n (μmol CO_2 m^2 s^{-1})
475
476
477
478
479
480
pTNVS299
pTKAW40
pTKAW39
Azygous

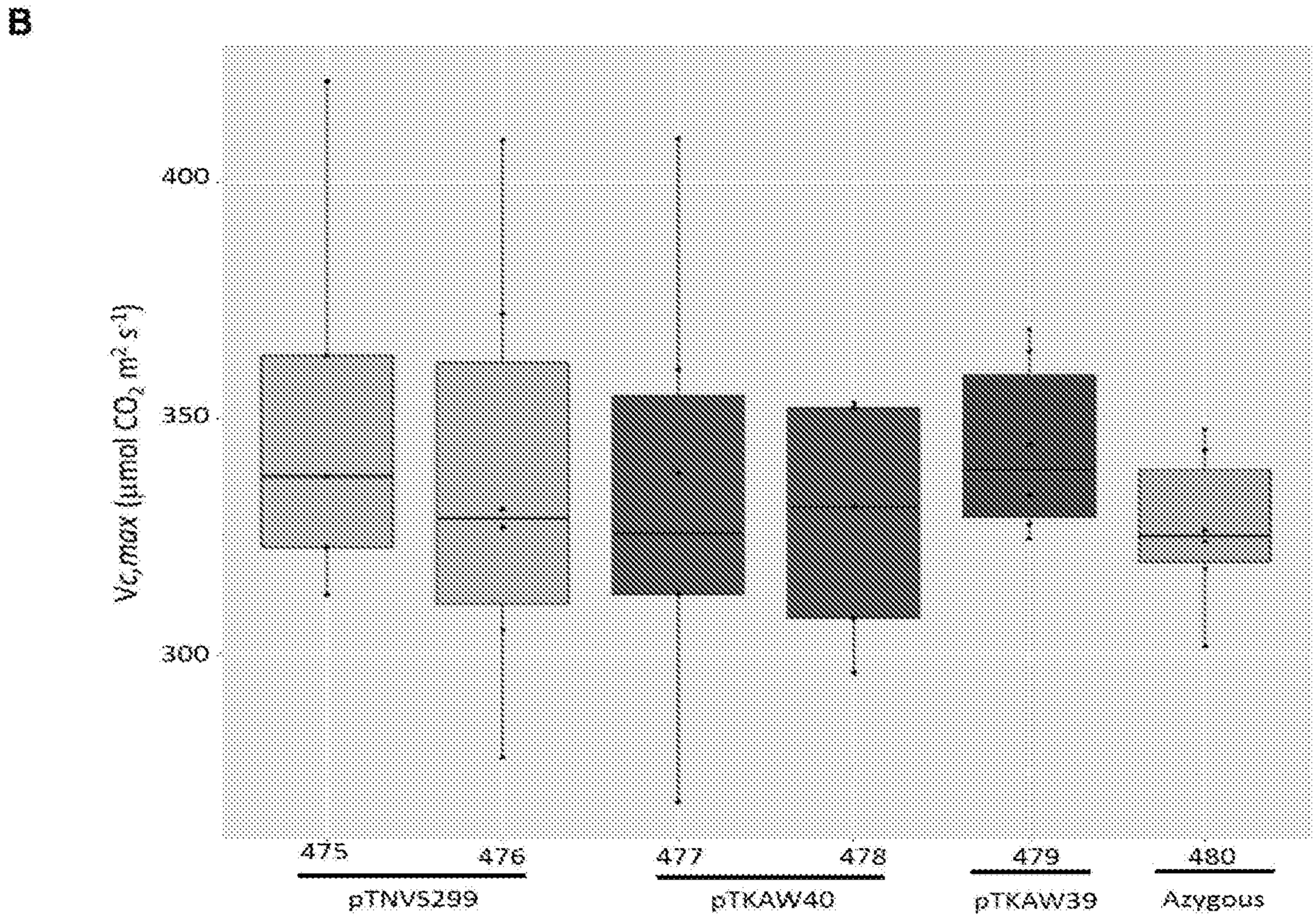

B
400
350
300
$V_{c,max}$ (μmol CO_2 m^2 s^{-1})
475
476
477
478
479
480
pTNVS299
pTKAW40
pTKAW39
Azygous

Figure 24

C

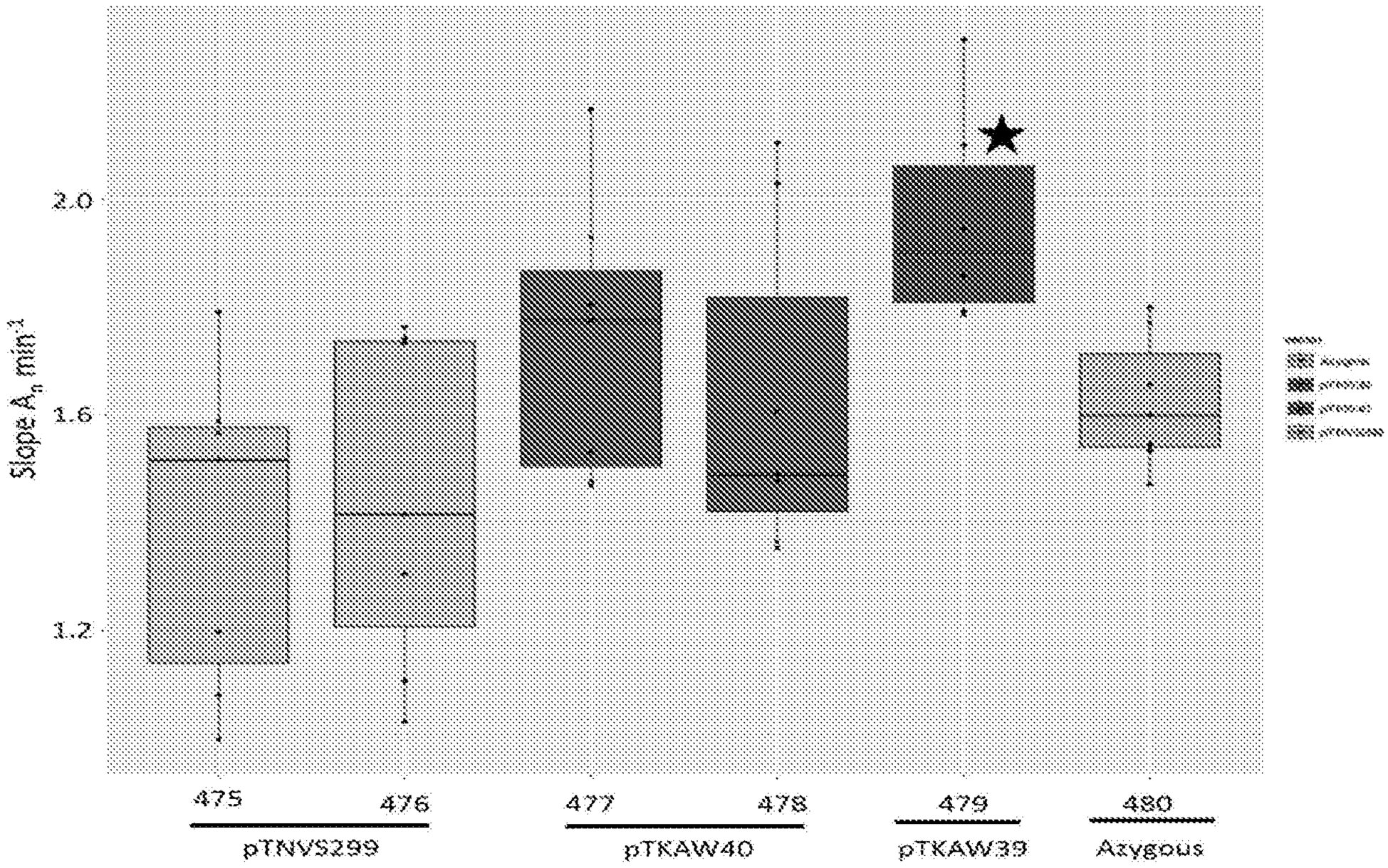


Figure 24 cont'd

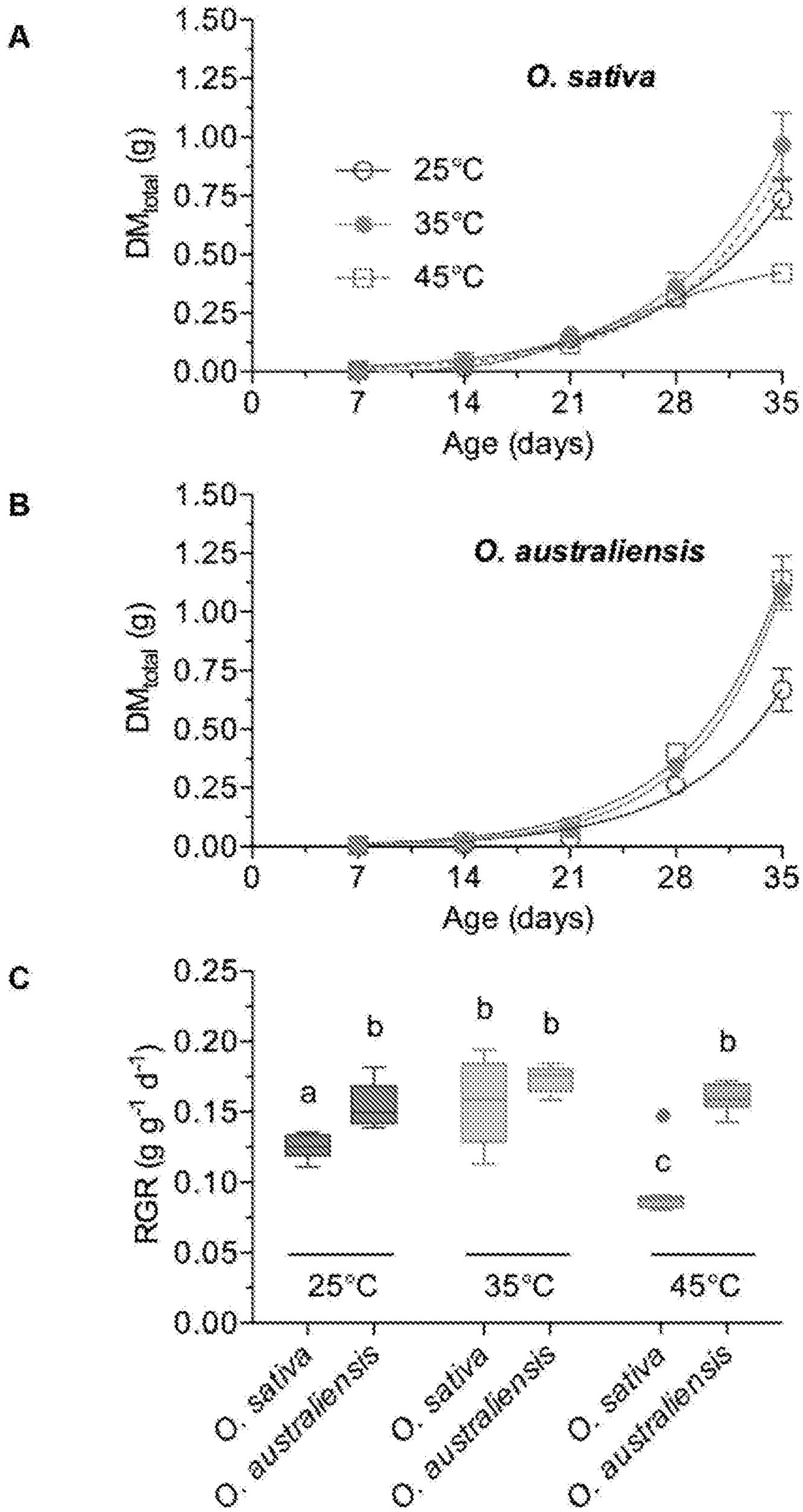

A
O. sativa
DM_{total} (g)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
25°C
35°C
45°C
0
7
14
21
28
35
Age (days)
B
O. australiensis
DM_{total} (g)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
7
14
21
28
35
Age (days)
C
RGR (g g^{-1} d^{-1})
0.25
0.20
0.15
0.10
0.05
0.00
a
b
b
b
c
b
25°C
35°C
45°C
O. sativa
O. australiensis
O. sativa
O. australiensis
O. sativa
O. australiensis

Figure 25

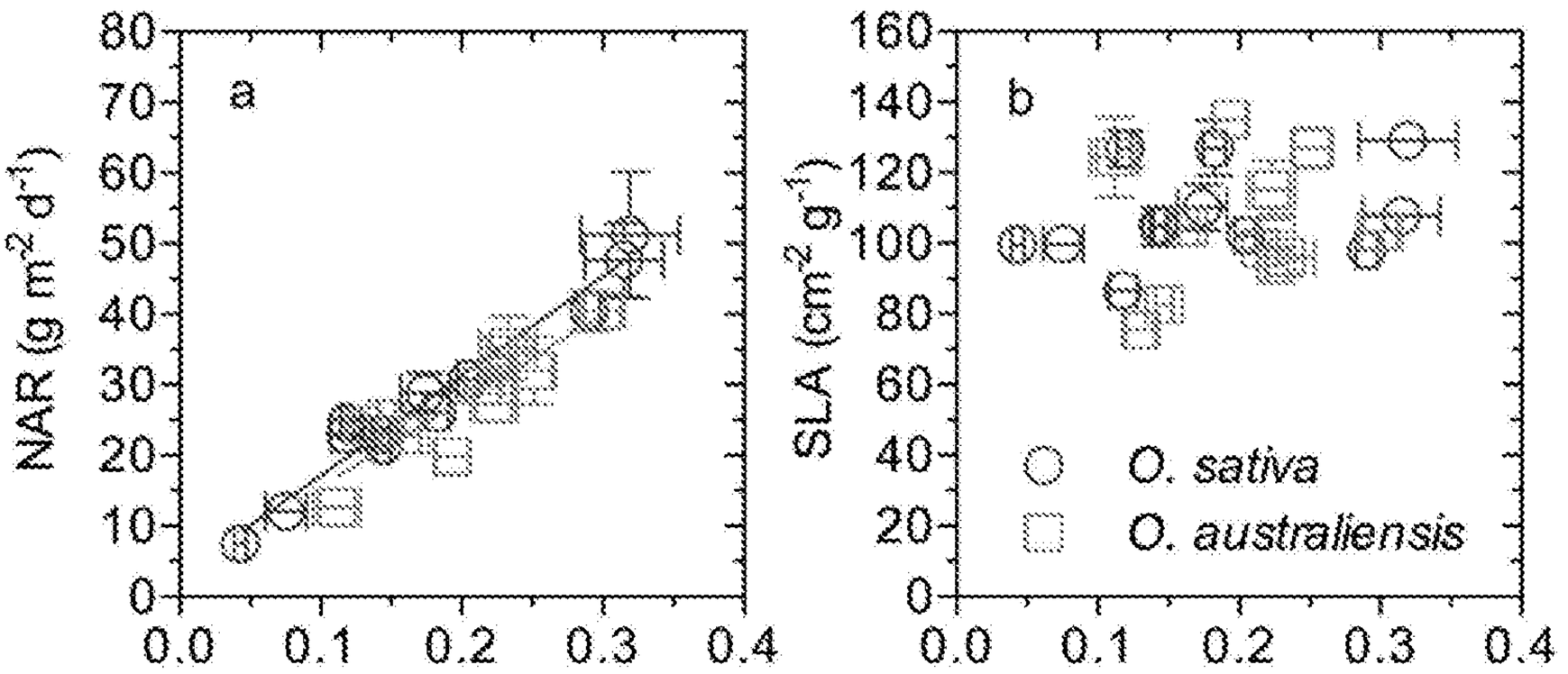


Figure 26

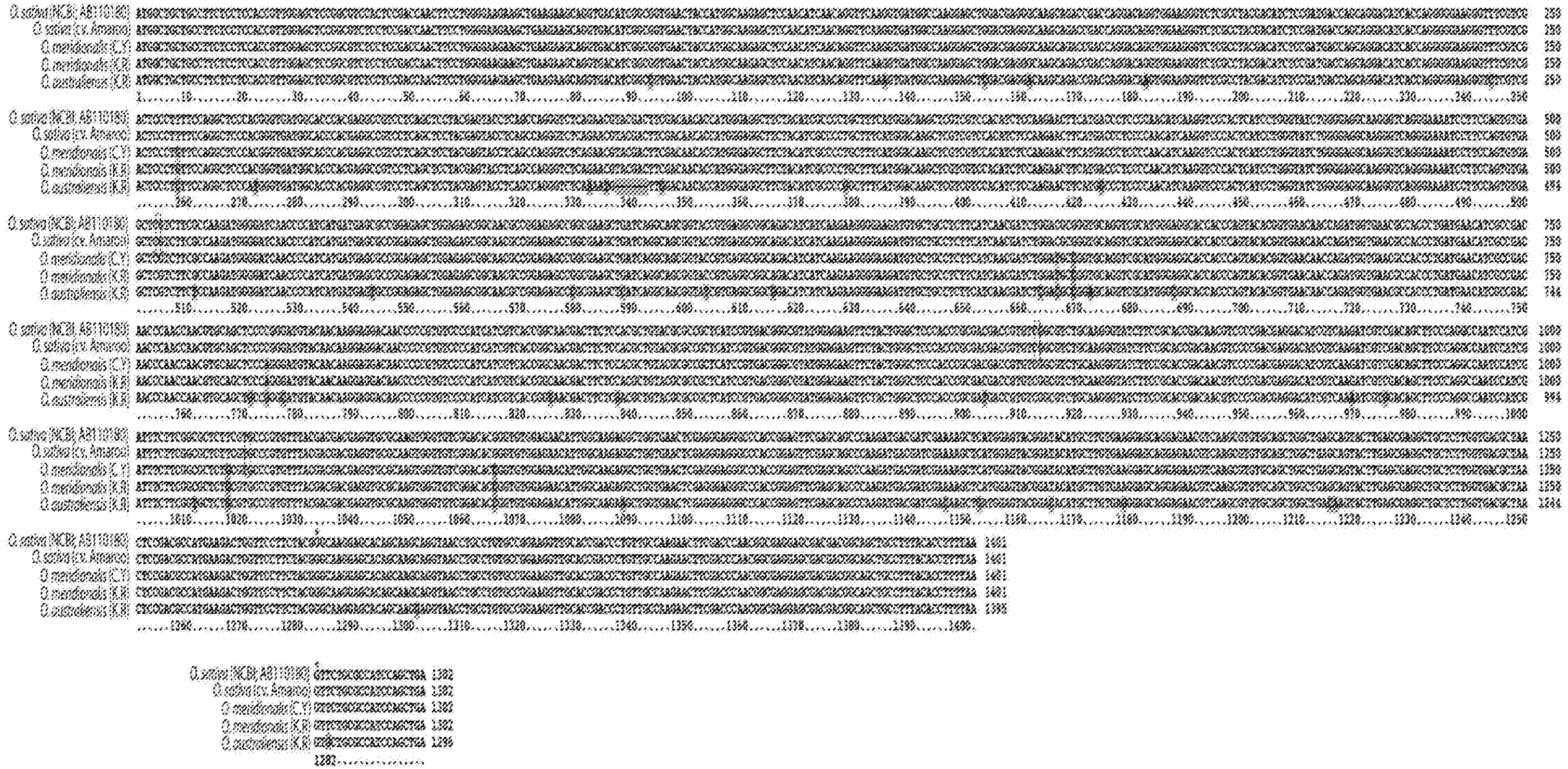


Figure 27

SUBSTITUTE SHEET (RULE 26)

Figure 28

SUBSTITUTE SHEET (RULE 26)

O. sativa
O. australiensis
G. hirsutum
L. tridentata
S. oleracea
O. antarctica

Figure 29

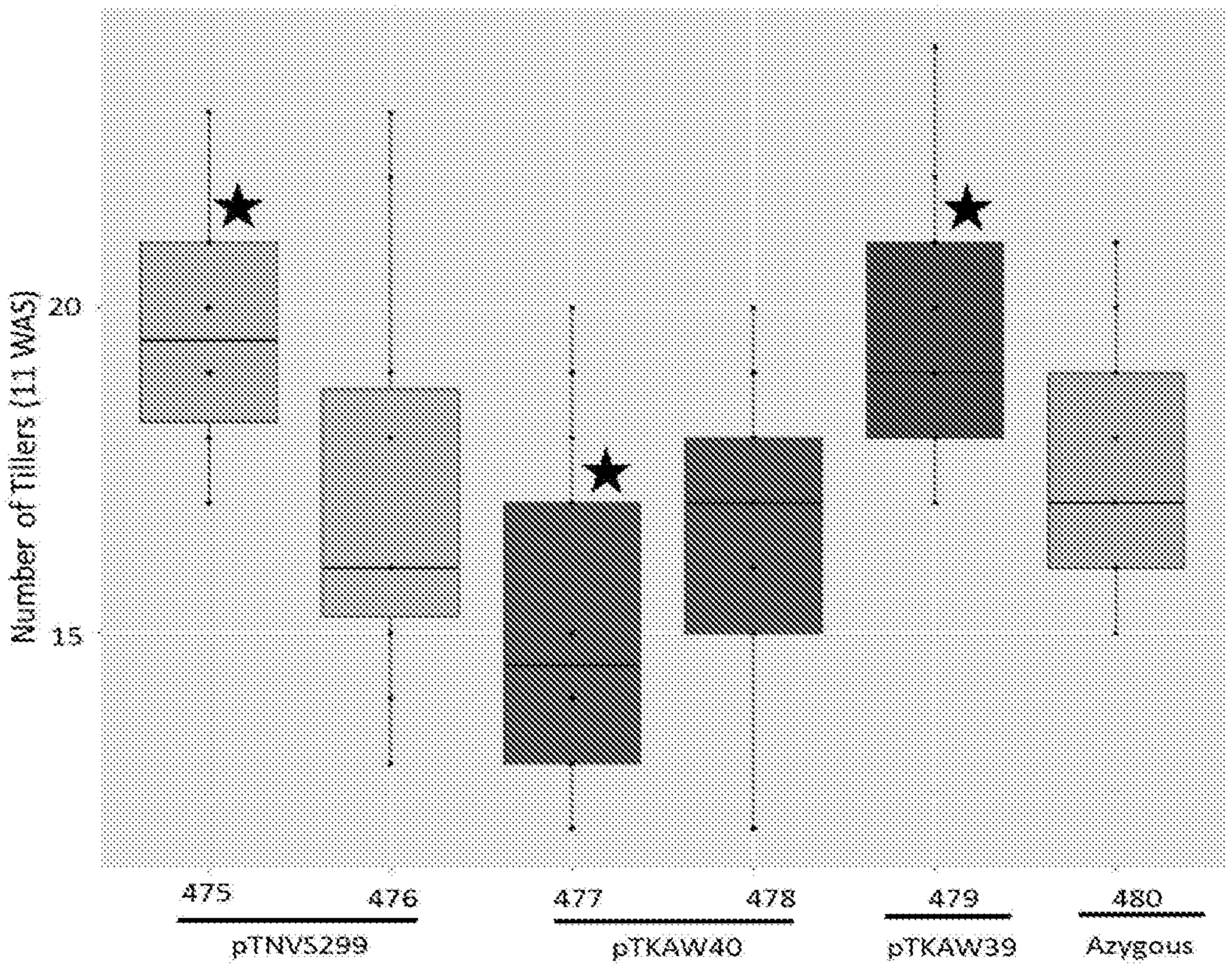


Figure 30

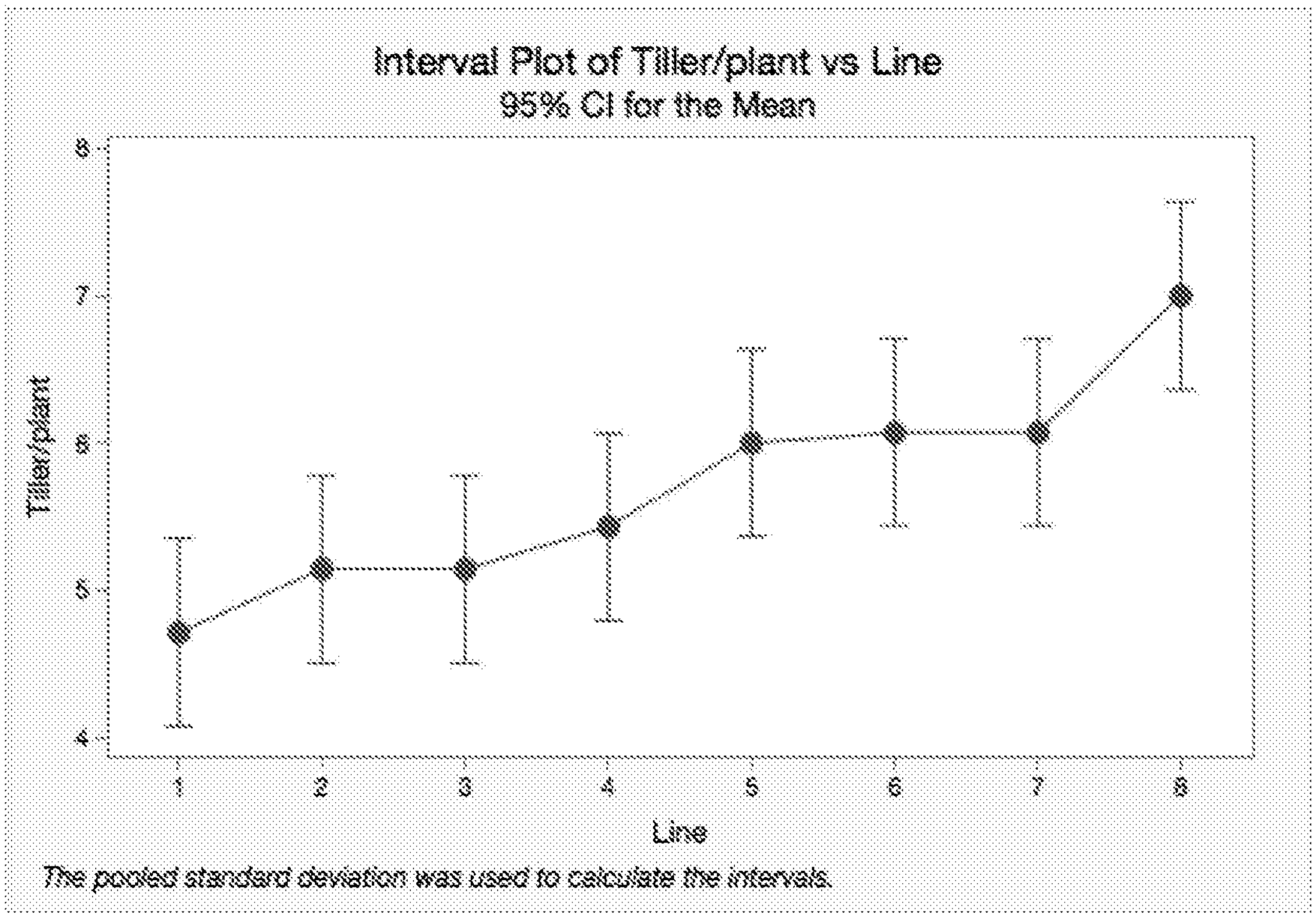


Figure 31