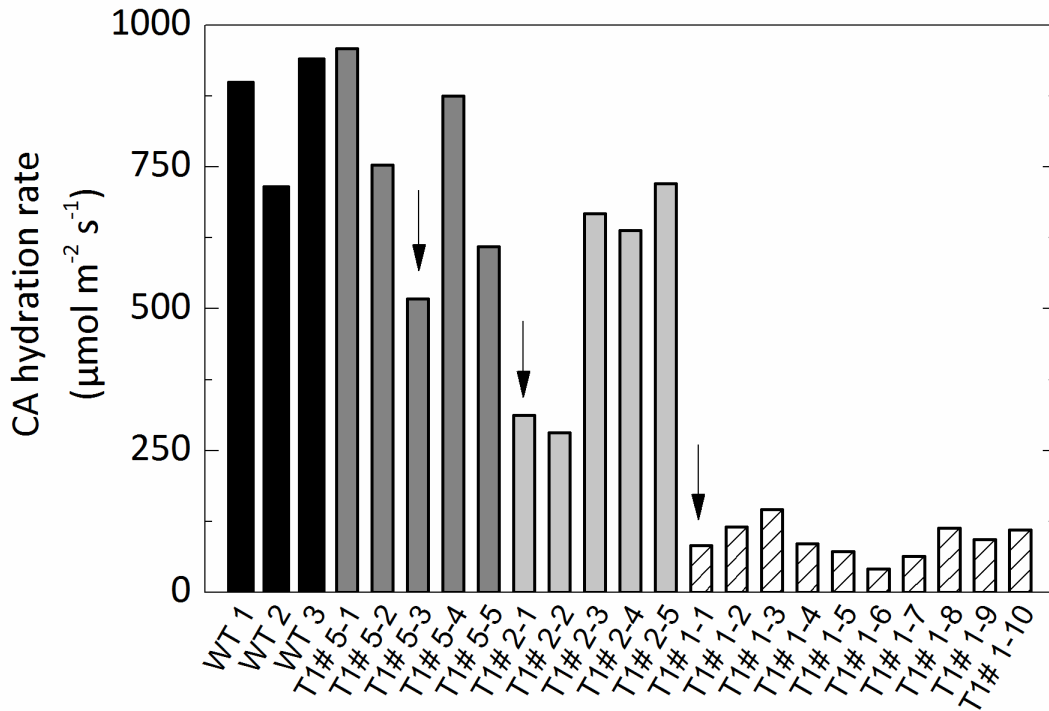
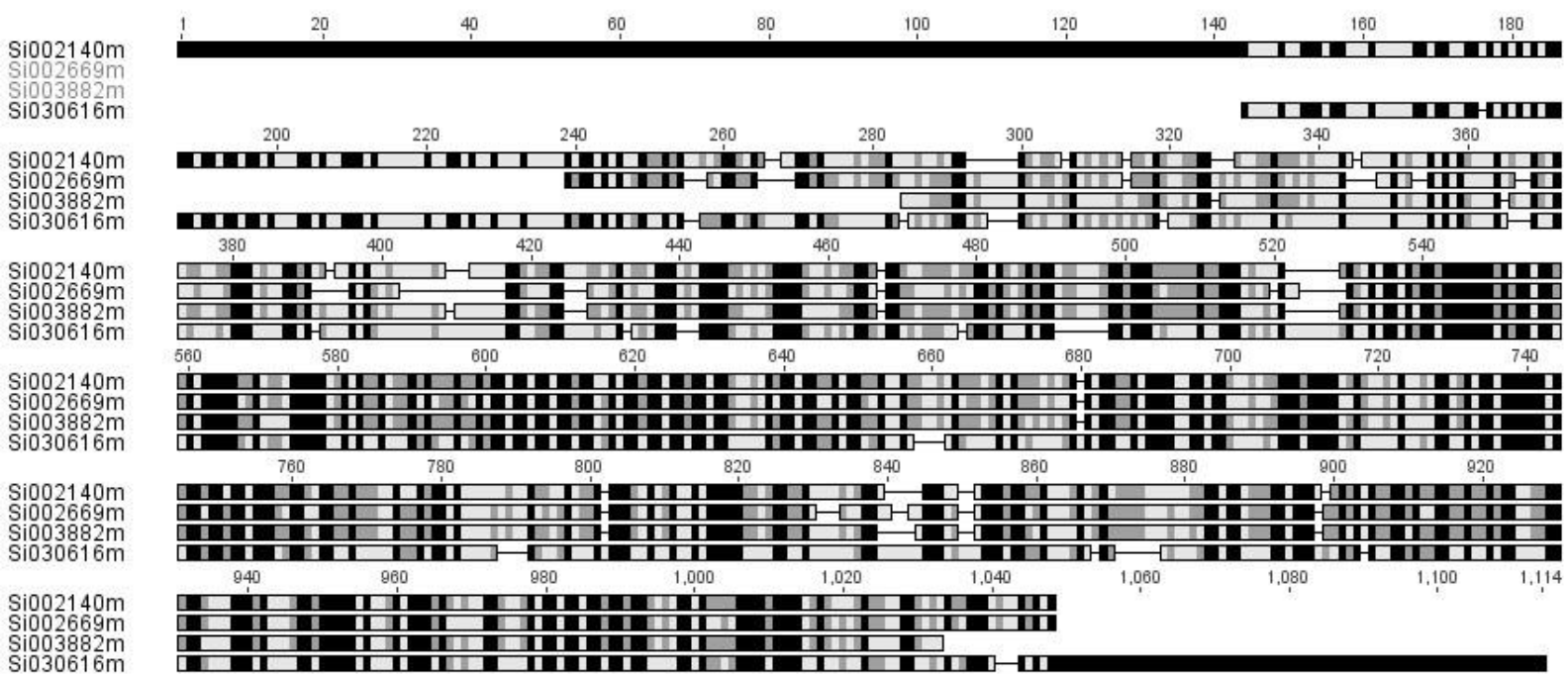


Supplementary Table 1: Primers used in this study.

Gene	Description	Sequence 5' - 3'	Amplicon Size (bp)	Use
Si016746m	Ubiquitin	F: GATCTCCGCCCCAGCAAGAT R: ATGCCCTCCTTGTCTGGAT	124	RT-qPCR housekeeping
Si022040m	Elongation factor 1a	F: GCTGCAACAAGATGGATGCC R: CCAGAGATTGGGACGAAGGC	132	RT-qPCR housekeeping
Si035709m	Beta tubulin	F: CTAAAGCTCGCCACCCCTAC R: GTCGGAGTTGAGCTGACCAG	104	RT-qPCR housekeeping
Si030616m	β CA	F: AGATGATTCAACCTCTGGAAGCT R: TTGCACTGCATTTCAAACCTCA	110	RT-qPCR
Si002140m	β CA	F: AGGCCGACAAGTTCCACTTC R: CATTGGTCCTCGAAAGCAGC	102	RT-qPCR
Si002669m	β CA	F: GGCTGGGTTTCAGGACGTTTA R: AGAGTCAGAGCACGCAAACA	112	RT-qPCR
Si003882m	β CA	F: CATAAATTCCTCCGCCTCGT R: CTCTCAAGCGCTCGACGG	101	RT-qPCR
Si003882m	β CA	F: GAGGGCCAGGCACCCAGGTA R: GACGGCGTACTCGATGGCGG	177	pSG/CAa insert
	Hygromycin phosphotransferase	F: TGGCGTGATTTTCATATGCGC R: CGTCAACCAAGCTCTGATAG	420	Genotyping
GRMZM2G348512	ZmCA2	F: CACCATGGACGACCCCGTCGAGCGCTTGAAGGAC R: TCAAGACCAGCCGCTCGCATCTTTCCAAGACGATG GCTGCTTATTGTCC	625	pSC110/ZmCA2 insert



Supplementary Figure 1: CA hydration rates at mesophyll $p\text{CO}_2$ in the T_1 plants from three transformation events. T_0 lines #5 and #2 were generated using the RNAi vector pSG/CAa. T_0 line #1 was generated by gene suppression with pSC110/*ZmCA2*. Arrows indicate the T_1 plants with low CA hydration rates whose progeny were then used for future studies and are labelled T1#5-3, T1#2-1 and T1#1-1.



Supplementary Figure 2: Very low DNA sequence identity (~37%) between the four main *Setaria* β -CAs using Clustal-W alignment.

pSC110/ZmCA2
 Si003882m
 ATGAGCGGGTGCCTTTGCCCTCCC TAAAAAGAAGGAGGGGGTCTCCGCAGCGAAGCAGTCCA TAAAT TCC

pSC110/ZmCA2
 Si003882m
 CCCGCCTCGTCACTCCTCCAGAACCAGAAGCCACCCAACCTCCACCTCCTCCT TCCAAGAAGGGC **ATG**

pSC110/ZmCA2
 Si003882m
GACGACC **CCGTCGAGCGCTTGAAG** GA **GGGTTTC** **ACAAGTTCAAGACCGA** G **GTCTATGACAAGAAGCCG**
GACC --- **CCGTCGAGCGCTTGAAG** AG **GGGTTT** G **ACAAGTTCAAGACCGA** T **GTCTATGACAAGAAGCCG**

pSC110/ZmCA2
 Si003882m
GAGCTGTTCCGAGCC **CTCAAGC** C **EGCCAGGC** **CCCA** A **GTAC** G **TGGTGT** T **CCCTGCTCCGACTCCCG** C
GAGCTGTTCCGAGCC **CTCAAGC** A G **EGCCAGGC** **CCCA** S **GTAC** A **TGGTGT** T **CCCTGCTCCGACTCCCG** T

pSC110/ZmCA2
 Si003882m
 GTG **TGCCCCGTCCGTTGACCC** **TGGGCCTGCAGCCCGGGGAGGCCTTCACCGTCCCGCAACATCCGCCGCCATG**
 TGC **TGCCCCGTCCGTTGACCC** **TGGGCCTGCAGCCCGGGGAGGCCTTCACCGTCCCGCAACATCCGCCGCCATG**

pSC110/ZmCA2
 Si003882m
GTCCCA G S **TACGACAAGACCAAGTACACCGGCAT** **GGTCCGCCATCCGAGTACGCCGT** T **TGCCG** S **CTC**
GTCCCA C S **TACGACAAGACCAAGTACACCGGCAT** **GGTCCGCCATCCGAGTACGCCGT** C **TGCCG** S **CTC**

pSC110/ZmCA2
 Si003882m
AAGGTGGAGGTCCTC G TG **GTCA** T **EGCCACAGC** T **CTGCGCGTGGCATCA** G **EGCGTCCCTCTCC** C **TCCAG**
AAGGTGGAGGTCCTC ACC **GTCA** T **EGCCACAGC** C **CTGCGCGTGGCATCA** A **EGCGTCCCTCTCC** A **TGAAG**

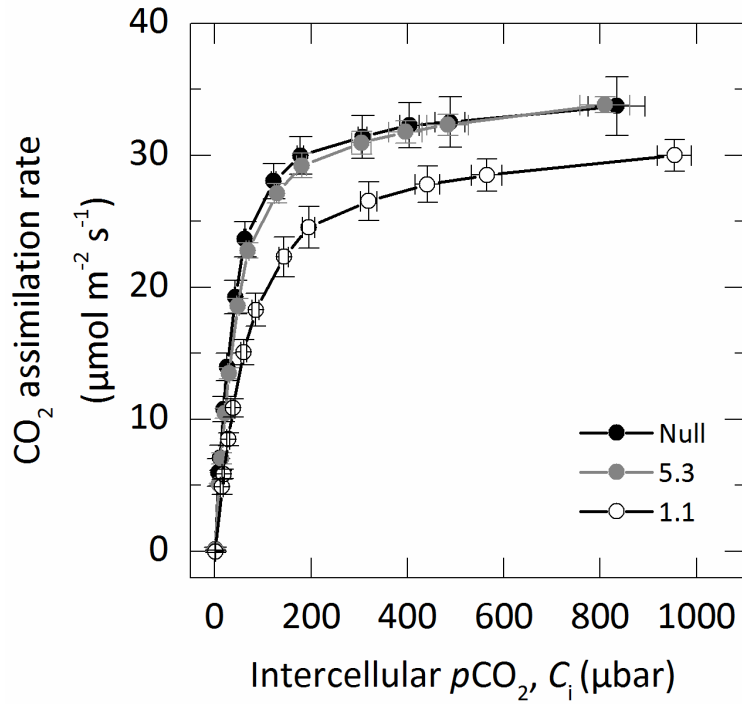
pSC110/ZmCA2
 Si003882m
CACGGCCACCTGACAAC **TCCACTTCGT** T **GAGAAC** **TGGCTTAA** **CA** **TCCGCTTCC** C T **CCCAAGA** T **TAA**
CACGGCCACCTGACAAC **TCCACTTCGT** S **GAGAAC** **TGGCTTAA** **CA** **TCCGCTTCC** T C **CCCAAGA** A **GAA**

pSC110/ZmCA2
 Si003882m
GTG AA **GA** **AAG** **ACCA** **CC** A **TGGGT** G **CCGTTCCGATGACCAGTCC** T **TA** **TTC** **TAGAGA** **AGGAGGCCGT** **TAA**
GTG C T **CA** **CA** **ACCA** **CC** C **TGGGT** T **CCGTTCCGATGACCAGTCC** A **CC** T C T **TAGAGA** **AGGAGGCCGT** **TAA**

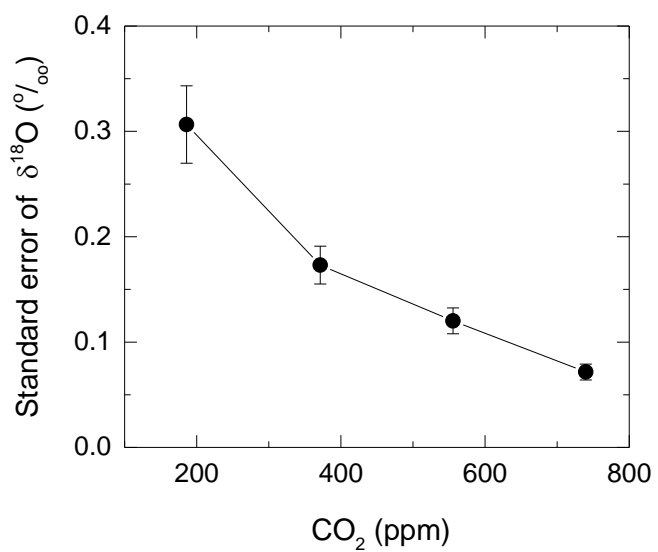
pSC110/ZmCA2
 Si003882m
 C **TGTC** **CCCT** **GA** S **AACCTC** AA **CACCTACCCCT** T C **GT** **AAGGA** **AGG** S C **TGGCCA** **ACGGGAC** G **GTCA** **AGCTG**
 G **TGTC** **CCCT** C T A **AACCTC** C T **CACCTACCCCT** G G **GT** **AAGGA** **AGG** T G **TGGCCA** **ACGGGAC** A **GTCA** **AGCTG**

pSC110/ZmCA2
 Si003882m
GTGGCGGGCCACTAC **AAC** **TTCGT** **GTCA** **GGG** **A** **TTTC** **CT** **ACATGGGACA** **ATAAGCAGCCATCGT**
GTGGCGGGCCACTAC **GAC** **TTCGT** **CAAC** **GGG** **T** **TTTC** **TC**

Supplementary Figure 3: High sequence identity (87%) of Si003882 to the *ZmCA2* (GRMZM2G348512) used for transformation in overexpression construct pSC110/ZmCA2 using Clustal-W alignment.



Supplementary Figure 4: CO_2 assimilation rate of transformed lines over a range of intercellular $p\text{CO}_2$ (C_i). Average of three T_2 plants of lines 5.3 and 1.1 were grown for TDL measurements. Plants were grown at 2 % CO_2 and the uppermost, fully expanded leaf of 5 week old plants were measured using a Li6400 at 25 °C leaf temperature at an irradiance of 1500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.



Supplementary Figure 5: Standard error of $\delta^{18}\text{O}$ in the reference gas of repeated measurements on the TGA200A. Each point represents the average \pm s.e. of standard errors calculated from eight different experiments.