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*Full Length Research Paper*

# A role for stomata in regulating water use efficiency in *Populus×euramericana* and characterization of a related gene, *PdERECTA*

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**The physiological mechanism of water use efficiency (WUE) remains elucidated, especially in poplar. We studied WUEi (instantaneous leaf transpiration efficiency), WUEL (ratio of unused biomass and water), carbon isotope composition ($\delta^{13}C$), photosynthetic rates (Pn), stomatal density and stomatal conductance (Gs) in three different, randomly chosen *Populus×euramericana* clones: DN-2 (*Populus × euramericana*), R-270 (*Populus×euramericana*) and NE-19 (*Populus nigra×*(*Populus×euramericana*)) under well-watered conditions. The clones had great differences in WUEL, WUEi, $\delta^{13}C$, stomatal density and Gs. We concluded that, stomatal control was a key factor leading to Pn and WUEi differences and ultimately inducing WUEL and $\delta^{13}C$ differences. $\delta^{13}C$ is therefore, a good indicator to use when evaluating WUEL in *Populus×euramericana*. We also went further to clone the *ERECTA* gene, which regulates plant transpiration efficiency in *Arabidopsis* from *Populus×euramericana.* Real-time polymerase chain reaction amplification (PCR) analysis revealed that, *PdERECTA* may play the same role in *Populus×euramericana* as it does in Arabidopsis.**

**Key words:** Carbon isotope composition, *Populus×euramericana*, PdERECTA gene, stomata, water use efficiency.

## INTRODUCTION

Agriculture is responsible for 70% of our water consumption. According to the International Water Management Institute, agricultural water use will increase by 17% in 2025. However, competition from urbanization and development means that, less water will be available for agriculture. Climate change also poses concerns for agronomists; rainfall in some areas is declining and becoming more erratic, wreaking havoc on markets (Bai et al., 2007; Aguirrezabal et al., 2006; Pennies, 2008). Therefore, much effort is being made to reduce water use by crops and to produce 'more crops per drop.

One of the most significant water-saving strategies is plant water use efficiency (WUE) (Galle et al., 2007). WUE varies among species, cultivars and populations; for example, in wheat (Farquhar and Richards, 1984), sugar beet (Rytter, 2005) and grapes (Boyer et al., 1997). Despite its obvious interest, the physiological mechanism of WUE remains to be elucidated. It depends on complex arrangements and interactions of physiological mechanisms such as stomatal behavior, photosynthetic type, photosynthetic capacity and carbon isotope composition ($\delta^{13}C$) (Aguirrezabal et al., 2006; Bray, 1997; Beerling and Chaloner, 1993; Ceulemans et al., 1995; Condon et al., 1990; Cowan and Farquhar, 1977; Specht et al., 2001).

*Populus×euramericana* is one of the most important species in large-scale forestation projects in China. However, its high water consumption is a key limiting factor in arid and semi-arid areas. Plant water use efficiency is influenced by both internal factors and the external envi-

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**Abbreviations: WUE,** Water user efficiency; **WUEi,** leaf-level water use efficiency; **WUEL,** crop water-use efficiency or yield; **Gs,** stomatal conductance; **Pn,** photosynthetic rates; **$\delta^{13}C$,** carbon isotopic composition; **PCR,** polymerase chain reaction amplification.

ronment (Ghannoum et al., 2003). Our interest has focused on determining the internal physiological mechanism of WUE. Our experiments were therefore performed under well-watered conditions to avoid stresses from the external environment.

Although, WUE can be studied by many strategies, little is known about the genetic control of transpiration efficiency (Braun et al., 1991; Chaves et al., 2003; Chang et al., 1992; Cominelli et al., 2005). The ERECTA (Keiko et al., 1996) has effects on stomatal density, epidermal cell expansion, mesophyll cell proliferation and cell to cell contact (Masle et al., 2005). It can change both stomatal number and leaf structure, thus, regulating plant transpiration rate and WUE and therefore, has excellent prospects for improving crop drought resistance and increasing WUE. In this study, we isolated and characterized an *ERECTA* cDNA clone, PdERECTA, which may play the same role in *Populus×euramericana* clones as in Arabidopsis.

## MATERIALS AND METHODS

### Plant material and growth conditions

The experiment was carried out in the greenhouse of Beijing Forest University. *Populus×euramericana* clones used were DN-2 (*Populus×euramericana*), R-270 (*Populus×euramericana),* and NE-19 (*Populus nigra* × (*Populus* × *euramericana*)). *Populus* × *euramericana* clones were planted in April 2008 and grown for 2 months under well-watered conditions. (80 to 100% of saturated soil water content). When the plants were about 30 cm tall, the water supply was controlled. All the clones were watered at 8:00 to 9:00 am and were fertilized once a month to fully supply nutrition. Every treatment was repeated five times.

### Physiological analyses

#### *Photosynthetic rate (Pn) and WUEi*

Rates of $CO_2$ assimilation (A) and transpiration (E) were measured by Li-6400. A whole-leaf clamp-on chamber was set up and measurements were made on a fully expanded mature leaf (9 to $12^{th}$ leaf from the top of the plant). Instantaneous leaf transpiration efficiency was calculated as the ratio A/E and termed *WUEi*.

#### *WUEL*

WUEL is the ratio of unused biomass and water in the period of controlling water. After the experiment, plants from all harvested cuttings were used for WUEL analysis. Samples were oven-dried for 24 h at 80°C and weighed on a precise scale.

### Stomatal density

Mature leaves (leaves used for gas exchange or of similar age: 3 to 5 replicate leaves per line) were cleaned and mounted on a light microscope fitted with a video camera. Measurements of stomatal density were performed on three small areas (36 to 38 × $10^3$ $\mu m^2$) selected on each side of the leaf blade, symmetrical along the main vein. Before clearing, a small section was cut out mid-way along the blade, fixed in 2.5% (v/v) glutaraldehyde, post-fixed in 1% (v/v) osmium tetroxide in 25 ml Mphosphate buffer pH7.0, slowly dehydrated in ethanol and embedded in Spurr's resin. Cross-sections were stained with toluidine blue and mounted on a light microscope fitted with a CCD camera for observation of mesophyll anatomy.

### Carbon isotope analysis

Leaf samples from all harvested cuttings were oven-dried for 24 h at 80°C and homogenized by grinding in a ball mill. Stable carbon isotope abundance in combusted samples was measured with a mass spectrometer (Finnegan MAT Delta-E, Bremen, Germany), as described by Hubick et al. (1986) and Li et al. (2000). The overall precision of the values was better than 0.1%, as determined from repeated samples. The entire analysis was performed in the Stable Isotope Laboratory for Ecological and Environmental Research, Chinese Academy of Sciences.

### Amplification of PdERECTA

Total RNA was extracted from *Populus×euramericana* clones. The cDNA of *Populus×euramericana* clones, PdERECTA, was cloned using a systematic reverse transcriptase (RT)-PCR-based strategy. cDNA syntheses were performed using the mRNA selective PCR Ver.1.1 kit (TaKaRa). The cDNA was amplified using the TaKaRa Ex Taq kit in a 50 µl volume, with 35 cycles of 94°C 1 min, 58°C 50 s and 72°C 1 min. PCR products were cloned into the pMD-18 vector (TaKaRa). The cDNA sequence was amplified by polymerase chain reaction amplification (PCR) using the primers P1 (5'-GTTCCTGT GAGCGAGTAATTC-3') and P2 (5'-GTCTTAATCGTATGCTGTAC ATCG-3'). The protein structure of PdERECTA was predicted using the www.smart.com website. We did not find any highly conserved genes in *Populus×euramericana*, but found one gene with a similar protein structure to ERECTA in Arabidopsis (data not shown).

### Real-time polymerase chain reaction amplification (PCR) and protein prediction analysis

Gene-specific primers for real time PCR were designed using DNAMAN. Each PCR primer amplified a region of approximately 150 bp. cDNA syntheses were performed using the Prime Script™ $1^{st}$ Strand cDNA synthesis kit (TaKaRa). Real-time PCR was performed using the power SYBR green PCR master mix kit (Applied Biosystems). PCR products were checked by melt-curve analysis and by agarose gel electrophoresis to ensure that only a single band of the expected size was amplified and that primer-dimer artifacts were absent. The expression level of the actin gene in *Populus×euramericana* clones was used as an internal control. The gene-specific primers were F (5'-GAATGCATCAAGCCAAACT GAGG-3'), R (5'-CAGCCGTAGTCGTGACAGTC-3'), Actin-F (5'-GTCCTCTTCCAGCCATC TC-3') and Actin-R (5'-TTCGGTCAGCA ATACCAGG-3').

## RESULTS

### WUEi and WUEL in three *Populus×euramericana clones*

For physiologists, the basic unit of production is moles of carbon gained in photosynthesis (A) in exchange for water

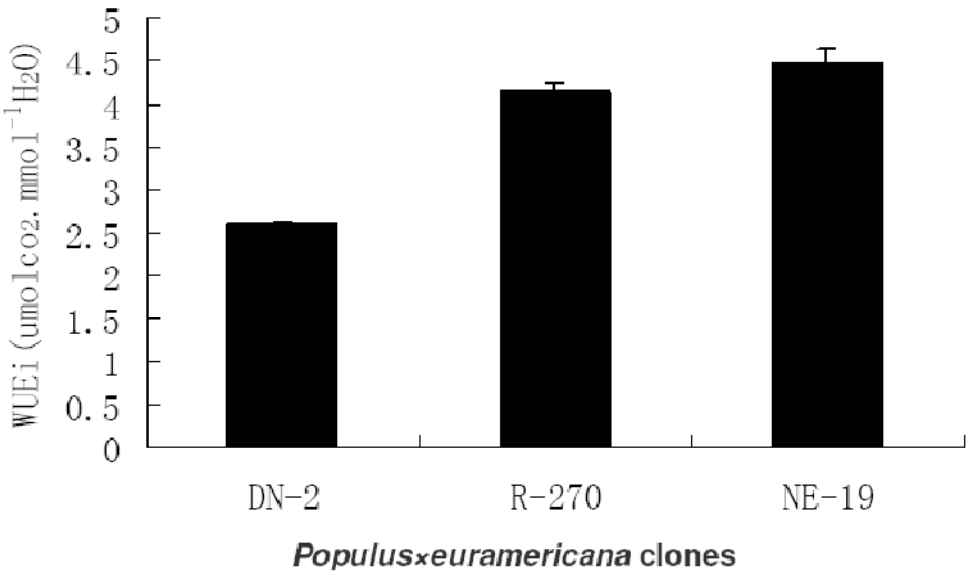


**Figure 1.** WUEi (Pn/Tr) of *Populus×euramericana* clones under well-watered conditions (three replications for each treatment).

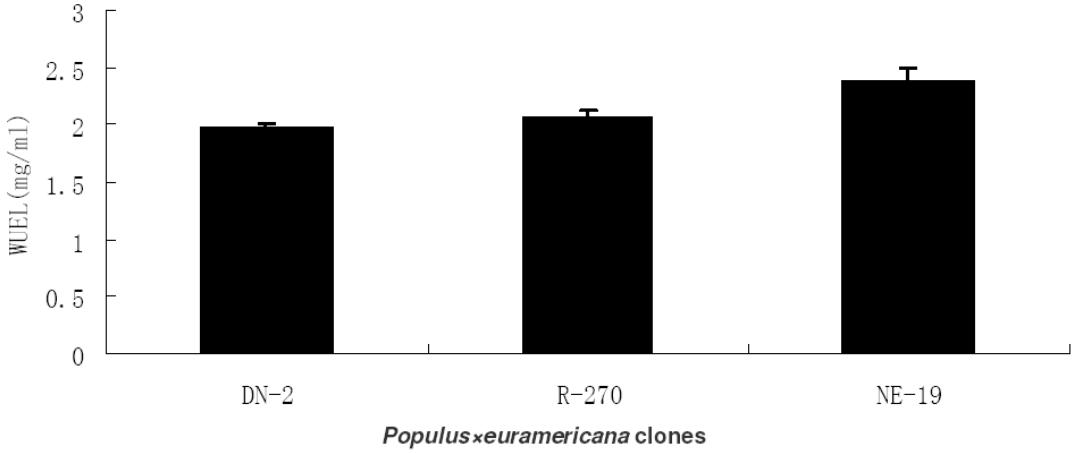


**Figure 2.** WUEL of *Populus×euramericana* clones under well-watered conditions (three replications for each treatment).

used in transpiration (T). Thus, a physiological definition might equate, at its most basic level, to the instantaneous water-use efficiency of leaf gas exchange (A/T, WUEi). In this study, the WUEi of R-270 (4.2 µmol $CO_2$.mmol$^{-1}H_2O$) was higher than that of DN-2 (2.6 µmol $CO_2$.mmol$^{-1}H_2O$) but lower than that of NE-19 (4.4 µmol $CO_2$.mmol$^{-1}H_2O$) (Figure 1).

For farmers and agronomists, the unit of production (WUEL) is much more likely to be the yield of harvested product achieved from the water made available to the crop through rainfall or irrigation. Therefore, we tried to determine the relationship between WUEi (leaf-level water use efficiency) and WUEL (crop water-use efficiency or yield). Experience shows that WUEi may not be always consistent with WUEL, because WUEL not only makes full use of water, but also has high rates of photosynthesis (Cutler et al., 1977). However in this study, we found that WUEi was consistent with WUEL. The WUEL of NE-19, R-270 and DN-2 were 2.41, 2.12 and 1.98 mg/ml, respectively (Figure 2).

## The relationship between WUE and $\delta^{13}C$

The stable isotope of carbon, $^{13}C$, makes up very close to 1% of the carbon in atmospheric $CO_2$. The proportion of $^{13}C$ in the dry matter of $C_3$ plants is fractionally less than that in the atmosphere, primarily because $C_3$ species

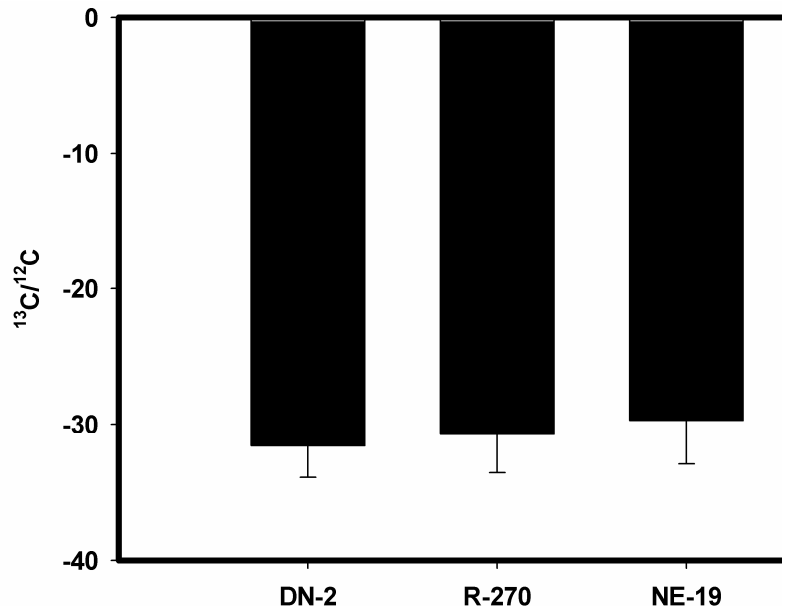


**Figure 3.** Carbon isotope composition (δ13C) of *Populus×euramericana* clones under well-watered conditions (three replications for each treatment).

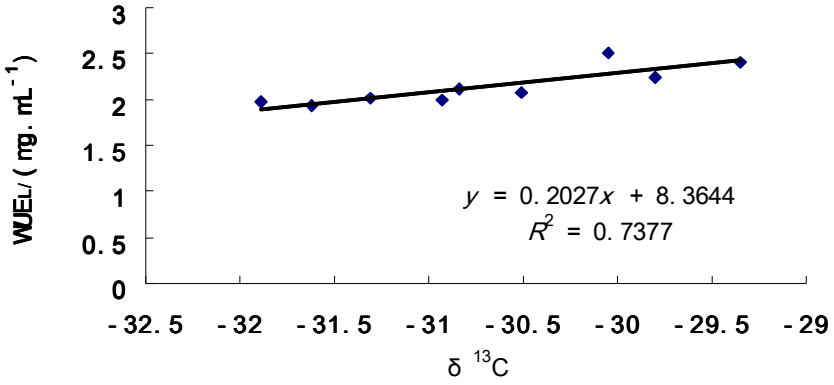


**Figure 4.** Correlation between carbon isotope composition and WUEL in *Populus×euramericana* clones under well-watered conditions.

discriminate against $^{13}C$ during photosynthesis. Carbon isotope discrimination ($\delta^{13}C$) is a measure of the $^{13}C/^{12}C$ ratio in plant material and is positively correlated with WUE. Therefore, $\delta^{13}C$ was examined to assess WUE in our study. The average $^{13}C/^{12}C$ of NE-19, R-270 and DN-2 was -29.732, -30.758 and -31.606, respectively (Figure 3). Also, we found that, $\delta^{13}C$ was well associated with WUE under well-watered conditions (Figure 4).

## Pn plays an important role in WUE differences

Photosynthesis is the basis of plant growth. High photosynthetic capacity means high accumulation of biomass (Wang et al., 2003). Therefore, Pn is an important parameter for plant WUE. Generally, plants with high WUEL also have high photosynthetic capacity. The Pn of NE-19, R-270 and DN-2 was 20.3, 20.5 and 20.8 μmol·m$^{-2}$·s$^{-1}$, respectively (Figure 5).

## Stomatal conductance and density are key factors in WUE differences of *Populus×euramericana* clones under well-watered conditions

Leaf transpiration efficiency describes the ratio of photosynthesis to transpiration rates. Clonal variation in transpiration efficiency could arise from variation in either of these components. To determine the mode of transpiration efficiency, we first examined its differences on stomatal conductance (Gs), a driver of transpiration rate.

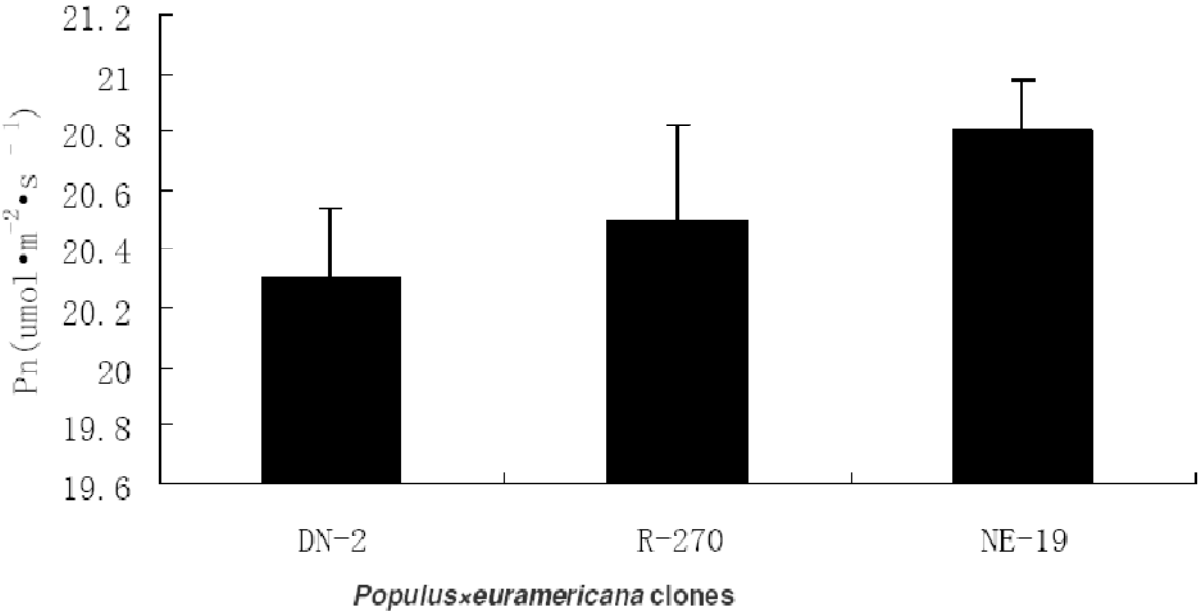


**Figure 5.** Trend of Pn in *Populus×euramericana* clones under well-watered conditions (three replications for each treatment).

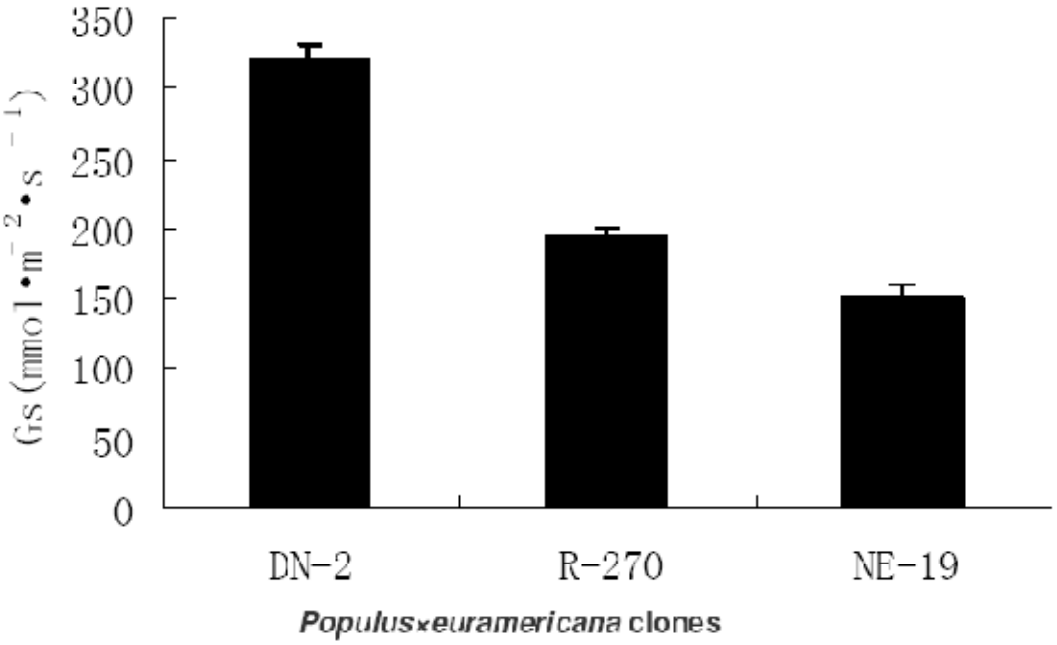


**Figure 6.** Comparison of Gs differences in *Populus×euramericana* clones under well-watered conditions.

Gs was obviously different among the *Populus × euramericana* clones. The Gs R-270 (190 mmol $m^{-2}$ $s^{-1}$) was lower than that of NE-19 (150 mmol $m^{-2}$ $s^{-1}$) and higher than that of DN-2 (320 mmol $m^{-2}$ $s^{-1}$) (Figure 6). We also examined the anatomy of the mature leaves. The stomatal density of R-270 was lower than that of NE-19 and higher than that of DN-2 (Figure 7).

## The expression of PdERECTA consists of WUE in *Populus×euramericana* clones

ERECTA is thought to be the first gene shown to coordinate transpiration and photosynthesis and therefore, to be identified as a transpiration efficiency gene. We cloned the *ER* gene from *Populus × euramericana*

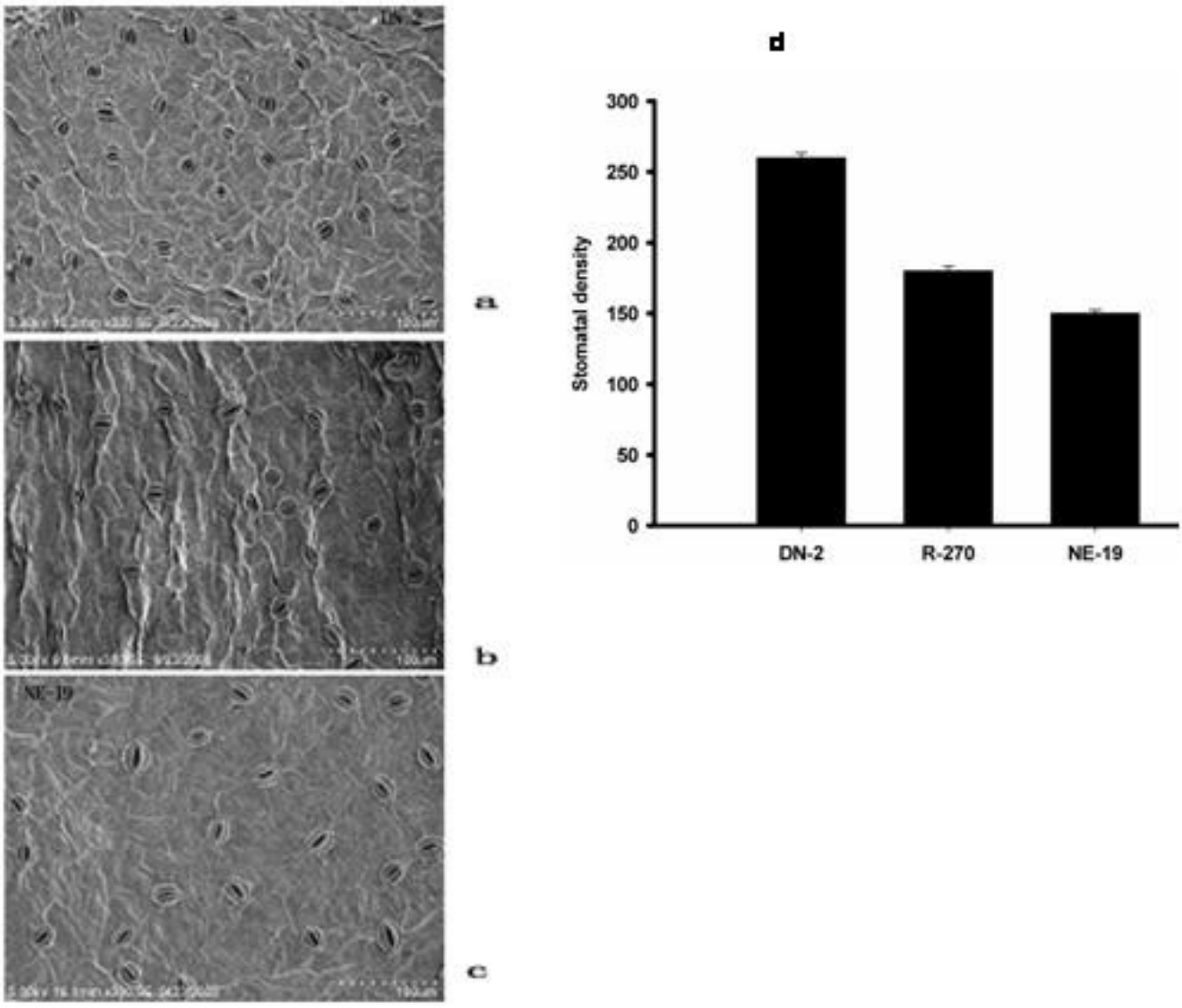


**Figure 7.** Comparison of stomatal density among three clones under sufficient water supply

*clones* and performed real time-PCR. Also, we found that, differences in PdERECTA expression were consistent with differences in WUE (Figure 8). This result indicates that, PdERECTA may play the same role in *Populus × euramericana* clones as in Arabidopsis.

## The *PdERECTA* gene encodes a putative receptor protein kinase with a ligand binding domain

The cDNA of PdERECTA is 2844 bp long and contains a single open reading frame of 948 amino acid residues. The deduced amino acid sequence of PdERECTA shows characteristics of a transmembrane receptor protein kinase with distinct domains (Figure 9). Two hydrophobic domains are present at the N terminus (amino acids 9 to 50) and between amino acids 551 and 573. These are consistent with a signal peptide and a transmembrane domain, respectively. The C-terminal cytoplasmic region (amino acids 617 to 883) comprises a putative catalytic domain of a protein kinase. A putative extracellular domain (amino acids 81 to 492) contains 10 tandem copies of a 24 amino acid LRR (Figure 10). These repeats have been implicated in protein-protein interactions. Protein serine/threonine and protein kinases are a group of enzymes that belong to a very extensive family of proteins, which share a conserved catalytic core common with both serine/threonine and tyrosine protein kinases. There are a number of conserved regions in the catalytic domain of protein kinases. In the N-terminal extremity of the catalytic domain, there is a glycine-rich stretch of residues in the vicinity of a lysine residue,

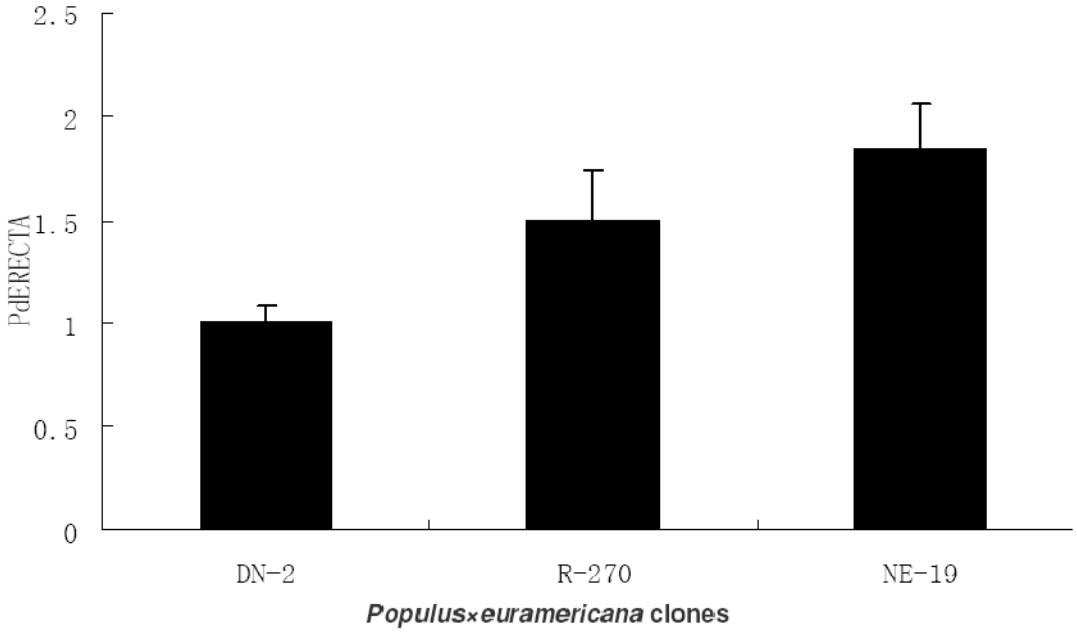


**Figure 8.** *PdERECTA* expression in *Populus×euramericana* clones.

which is involved in ATP binding. In the central part of the catalytic domain, there is a conserved aspartic acid residue that is important for the catalytic activity of the enzyme.

## DISCUSSION

### Carbon isotope discrimination is closely related to WUE in *Populus×euramericana* clones

The positive relationship between $\delta^{13}C$ and WUE in *Populus × euramericana* was only clearly observed under well-watered conditions and disappeared under serious stress (data not shown). $C_3$ plants have only one type of Rubisco carboxylase. Due to its high quantity and low weight, $^{12}C$ is easily absorbed by Rubisco carboxylase. Therefore, under well-watered conditions, any clones with low Gs that also have high Pn could make use of more $^{13}C$, and have a higher WUE. Although $\delta^{13}C$ is heritable, it is strongly modulated by environmental factors. Under water stress, $\delta^{13}C$ was increased, causing a change in stomatal structure. The relationship between $\delta^{13}C$ and WUE was reduced greatly, especially under severe stress, because the difference in $\delta^{13}C$ caused by water stress was much higher than that, due to genotypic differences.

### Stomatal density, Gs and Pn play an important role in regulating WUE in *Populus×euramericana* clones

The leaf stoma is a pivotal gate controlling the exchange of $CO_2$ and water vapor. Stomata play an important role in both $CO_2$ assimilation and water relations of plants (Galme et al., 2007; Wong et al., 1979). Therefore, stomatal traits have been suggested as criteria for selection of clones or genotypes that are more productive and have higher WUE. However, such processes may be affected by many environmental variables, including light, water status, temperature and $CO_2$ concentration (Braun et al., 1991; Wang et al., 2003; Wong et al., 1979). In our study, R-270 and NE-19 had high Pn, but they also had lower stomatal density and stomatal conductance. This lead to a decrease of water vapor and ultimately induced differences in WUEL and $\delta^{13}C$.

### *PdERECTA* gene may have the same role in *Populus×euramericana* and Arabidopsis

Real time-PCR analysis showed that the expression of the *PdERECTA* gene in *Populus×euramericana* clones was consistent with that of WUE. We suggest that the *PdERECTA* gene, which encodes a putative transmembrane receptor protein kinase with an extracellular ligand binding domain, may play the same role in the *Populus × euramericana* clones as it does in *Arabidopsis*. The protein kinases play an important role in a multitude of cellular processes, including division, proliferation, differentiation and apoptosis. Phosphorylation usually results in a functional change of the target protein by changing enzyme activity, cellular location or association with other proteins. The putative function of PdERECTA also strongly suggests that, it may participate in intercellular signal transduction, perhaps through interaction

MSIKESFSNVVNVLLDWDDVHNEDFCSWRGVFCDNVSLSVVSLNLSNLNLGGEISPAIGD

I

LRNLQSIDFKGNKLTGQIPEEIGNCASLFNLDLSDNLLYGDIPFSISKLKQLDTLNLKNN
QLTGPIPSTLTQIPNLKTLNLAKNQLTGEIPRLIYWNEVLQYLGLRGNLLTGTLSEDMCQ
LTGLWYFDVRGNNLSGTIPSSIGNCTSFEILDISYNQISGEIPYNIGFLQVATLSLQGNS
LTGKIPEVIGLMQALAVLDLSDNELVGPIPPILGNLSYTGKLYLHGNKLTGPIPPELGNM
SKLSYLQLNDNQLVGRIPPELGMLEQLFELNLANNHLEGPIPNNISSCRALNQLNVYGNH
LSGIIASGFKGLESLTYLNLSSNDFKGSIPIELGHIINLDTLDLSSNNFSGPIPASIGDL
EHLLILNLSRNHLHGRLPAEFGNLRSIQAIDMSFNNVTGSIPVELGQLQNIVTLILNNND
LQGEIPDQLTNCFSLANLNFSYNNLSGIVPPIRNLTRFPPDSFIGNPLLCGNWLGSVCGP

II

YVLKSKVIFSRAAVVCITLGFVTLLSMVVVVIYKSNQRKQLIMGSDKTLHGPPKLVVLHM

III

DIAIHTFDDIMRNTENLSEKYIIGYGASSTVYKCVLKNSRPLAIKRLYNQYPYNLHEFET
ELETIXSIRHRNIVSLHGYALSPRGNLLFYDYMKNGSLWDLLHGSSKKVKLDWETRLKVA
VGAAQGLAYLHHDCNPRIIHRDVKSSNILLDEDFEAHLSDFGIAKCIPTTKSHASTFVLG
TIGYIDPEYARTSRLTEKSDVYSFGIVLLELLTGKKAVDNESNLQQLILSRADDNTVMEA
VDPEVSVTCMDLTHVKKSFQLALLCTKRHPSERPTMQDVSRVLVSFLPALPTKASLLPKP
IDYAKFVIDKGQQQQPIVNQQQPSQENNSSDAQWFVRFKEVVSKNTL

**Figure 9.** *PdERECTA* encodes 948 amino acids. Region I, extracellular LRRs; II, transmembrane; III, cytoplasmic kinase domain.

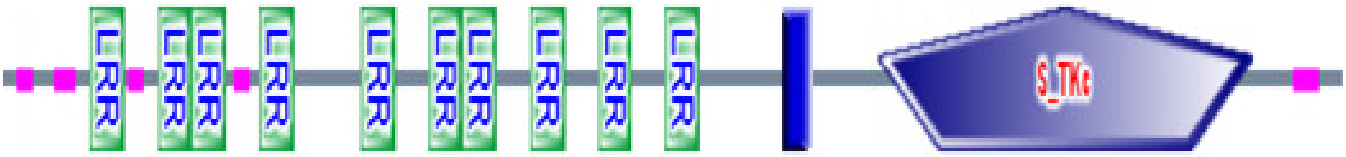


**Figure 10.** Deduced protein domain analysis of PdERECTA.

with extracellular ligands that activate the intracellular kinase domain and ultimately change the stomatal density just as ERECTA does in Arabidopsis. However, we do not know yet the pathway of gene expression. Therefore, more work is needed to confirm the function of the *PdERECTA* gene by transgenic analysis. If we can identify the potential target, characterize and tailor molecular mechanisms for tight stomatal adjustments and improved WUE, it will be helpful in future genetic improvements of WUE in crops grown in semi-arid and arid regions.

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