



Invited review

Molecular and physiological responses to abiotic stress in forest trees and their relevance to tree improvement

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Received July 16, 2013; accepted February 6, 2014; published online April 2, 2014; handling Editor Vaughan Hurry

Abiotic stresses, such as drought, salinity and cold, are the major environmental stresses that adversely affect tree growth and, thus, forest productivity, and play a major role in determining the geographic distribution of tree species. Tree responses and tolerance to abiotic stress are complex biological processes that are best analyzed at a systems level using genetic, genomic, metabolomic and phenomic approaches. This will expedite the dissection of stress-sensing and signaling networks to further support efficient genetic improvement programs. Enormous genetic diversity for stress tolerance exists within some forest-tree species, and due to advances in sequencing technologies the molecular genetic basis for this diversity has been rapidly unfolding in recent years. In addition, the use of emerging phenotyping technologies extends the suite of traits that can be measured and will provide us with a better understanding of stress tolerance. The elucidation of abiotic stress-tolerance mechanisms will allow for effective pyramiding of multiple tolerances in a single tree through genetic engineering. Here we review recent progress in the dissection of the molecular basis of abiotic stress tolerance in forest trees, with special emphasis on *Populus*, *Pinus*, *Picea*, *Eucalyptus* and *Quercus* spp. We also outline practices that will enable the deployment of trees engineered for abiotic stress tolerance to land owners. Finally, recommendations for future work are discussed.

Keywords: acclimation, epigenetic control, genetic variation, natural population, regulatory networks, signaling, stress, tolerance, tree growth.

Introduction

Forest trees constitute ~82% of the continental biomass (Roy et al. 2001) and harbor >50% of the terrestrial biodiversity (Neale and Kremer 2011). They also help to mitigate climate change, and provide a wide range of products that meet human needs, including wood, biomass, paper, fuel and biomaterials (Harfouche et al. 2012).

Field-grown trees are routinely exposed to environmental stress. Current and predicted climatic conditions, such as prolonged drought, increased salinization of soil and water, and low-temperature episodes, pose a serious threat to forest productivity

worldwide, affecting tree growth and survival. Because of global climate change, these stresses will increase in the near future, according to reports from the Intergovernmental Panel on Climate Change (<http://www.ipcc.ch>).

Conventional breeding has been successful at improving various traits that impact tree growth, such as crown and root architecture and partial resistance to biotic stress. Because of their long generation times, large genomes and the lack of well-characterized mutations for reverse-genetic approaches, continued improvement of forest trees by these means is slow (Neale and Kremer 2011). Nevertheless, over the past two decades, breeding possibilities have been broadened by

genetic engineering and genomic-based approaches targeting model forest-tree species (Harfouche et al. 2011, Tsai 2013, White et al. 2013). An understanding of how forest trees adapt to harsh environmental conditions is necessary in order to sustain productivity and meet future demands for commercial products. Current efforts to use molecular analysis and genetic engineering to improve abiotic stress tolerance depend on a detailed understanding of plant signaling pathways, as well as the genes that encode and regulate key proteins, as reviewed here.

Understanding the molecular and genetic basis of complex, polygenic traits is an important goal in plant genetic improvement, with wide-ranging effects across many disciplines and methodologies (Altman and Hasegawa 2012). The completion of a draft sequence of the poplar (*Populus trichocarpa* Torr. & Gray; Tuskan et al. 2006) genome has advanced forest-tree genetics to unprecedented levels. Recent releases of the white spruce [*Picea glauca* (Moench) Voss; Birol et al. 2013] and Norway spruce (*P. abies* L.; Nystedt et al. 2013) genome sequences and the anticipated release of the *Pinus* genome will undoubtedly lead to more rapid advances. Omics analyses allow us to decipher regulatory networks in plant abiotic stress responses (Urano et al. 2010), and indeed, several promising avenues of research in abiotic stress responses have been described in recent years. These include gene networks and upstream regulation, acclimation signaling networks, epigenetic control of gene expression during stress and systems-biology approaches. Transcriptomic, proteomic and metabolomic analyses have been used to identify and characterize several genes and expression products that are induced by drought, salinity and cold stresses, as well as their associated signaling and regulatory pathways in trees (Harfouche et al. 2012).

Opportunities and challenges involved in engineering trees for tolerance to abiotic stresses have been reviewed previously (Neale 2007, Yadav et al. 2010, Harfouche et al. 2011, Osakabe et al. 2012). Newer technologies, such as genomic selection (GS) and next-generation Ecotilling (Harfouche et al. 2012), are now providing additional approaches to increasing our understanding of various processes in forest trees and the role of the associated key genes (Marroni et al. 2011, M.F.R. Resende et al. 2012a, Vanholme et al. 2013). Although there have been significant advances in our understanding of abiotic stress-signal perception and transduction and of the associated molecular regulatory networks, especially in poplar (*Populus* spp.), most of the genetic engineering efforts have been restricted to poplars, and engineering for drought, salinity and cold tolerance in other forest trees is still in its infancy. Moreover, the number of transgenic trees that have been field-tested or evaluated under natural water-deficit conditions is very low. More tree transgenesis is urgently needed to link physiology, systems biology and field performance.

This review summarizes recent achievements in the study of the molecular responses to drought, salinity and cold, including sensing, downstream signaling pathways and outputs of stress-responsive genes, with reference to the various events that are outlined in Figure 1. We further review epigenetic responses to abiotic stress, GS and quantitative-trait loci (QTL) studies, and discuss issues that should be considered when developing trees with improved tolerance to environmental conditions.

Responses of forest trees to drought, salt and cold stress

Environmental stresses affect many aspects of tree physiology and metabolism, and can negatively impact tree growth, development and distribution. Stress responses and tolerance mechanisms involve the prevention or alleviation of cellular damage, the re-establishment of homeostasis and growth resumption (Figure 1). Because forest trees are sessile and continue to develop over many growing seasons, mechanisms have evolved that allow trees to respond to changes in environmental conditions. Adaptation to environmental stresses is controlled by molecular networks, and significant progress using transcriptomics, proteomics and metabolomics has facilitated discoveries of abiotic stress-associated genes and proteins involved in these cascades (Table 1). Some advances in identifying those stress-responsive mechanisms in forest trees are highlighted below.

Water deficit

As water potential declines, trees reduce transpiration by closing their stomata, but reducing water loss is done at the expense of CO₂ assimilation (Jarvis and Jarvis 1963, Cowan 1977). Although stomatal closure is efficient in limiting water loss (Froux et al. 2005), it cannot prevent water-deficit-related decline in trees. Water stress can disrupt water fluxes in the xylem, leading to cavitation. The resulting embolisms limit the plant's ability to conduct water, and thus limit tree growth (Tyree et al. 1994, Rice et al. 2004). The ability to avoid drought stress is dependent on the tree's ability to minimize loss and maximize uptake of water (Chaves et al. 2003). For example, some forest trees increase water uptake through deeper and more extensive root systems (Nguyen and Lamant 1989), modify a variety of leaf characteristics, including altered morphology (e.g., cuticular wax, Hadley and Smith 1990), and reduce leaf area (e.g., increased leaf abscission, Munné-Bosch and Alegre 2004) and stomatal conductance. Active accumulation of solutes in vacuoles (i.e., osmotic adjustment) is a common physiological response to drought, salinity and cold stress, depending among other mechanisms on shunting photosynthate from growth to tolerance and/or acclimation. This may be one of the reasons why the metabolic costs of stress tolerance may have negative impacts on growth.

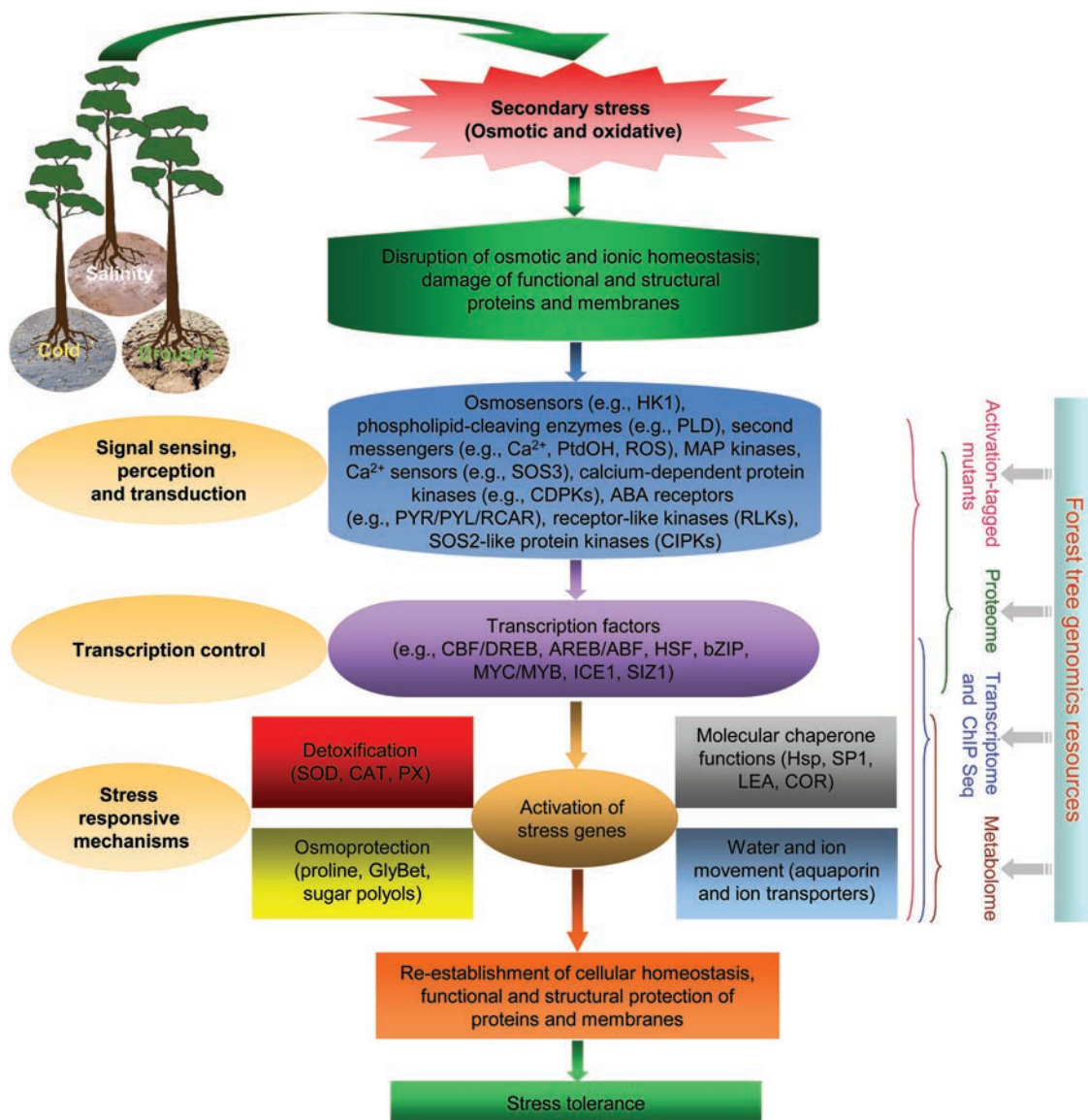


Figure 1. Overall model of abiotic stress responses. Primary stresses, such as drought, salinity and cold, are often interconnected and cause cellular damage and secondary stresses, such as osmotic and oxidative. The initial stress signals (e.g., osmotic and ionic effects or changes in temperature or membrane fluidity) trigger downstream signaling process(es) and transcriptional controls, which activate stress-responsive mechanisms to re-establish cellular homeostasis and to protect and repair damaged proteins and membranes. Inadequate responses at one or more steps in the signaling and stress-gene-activation process might ultimately result in irreversible changes in cellular homeostasis and in the destruction of functional and structural proteins and membranes, leading to cell death. ABF, ABA responsive element (ABRE) binding factor; bZIP, basic leucine zipper transcription factor; CAT, catalase; CBF/DREB, C-repeat-binding factor/dehydration-responsive-binding protein; CDPK, calcium-dependent protein kinase; CIPK, calcineurin B-like interacting protein kinase; COR, cold-responsive protein; HK1, histidine kinase-1; Hsp, heat-shock protein; ICE1, inducer of CBF expression 1; LEA, late embryogenesis abundant; MAP, mitogen-activated protein; PLD, phospholipase D; PtdOH, phosphatidic acid; PX, peroxidase; PYR, pyrabactin resistance; PYL, PYR-like; RCAR, regulatory component of ABA response; ROS, reactive oxygen species; SIZ1, SAP and Miz domain protein 1; SOS, salt overly sensitive; SOD, superoxide dismutase; and SP1, stable protein 1. The growing resources in forest-tree genomics have countlessly contributed to clarifying this complex system with regard to genetic and epigenetic regulation. Figure modified from Vinocur and Altman (2005).

Salinity stress

High soil salinity, frequently caused by excess NaCl (Zhu 2001, Apse and Blumwald 2002, Tester and Davenport 2003), imposes ion imbalance and hyper-osmotic stress, leading to cell membrane disorganization, ion toxicity and oxidative damage (Zhu 2001). Both glycophytes and halophytes have

evolved many adaptation mechanisms to cope with salt stress (Thomas and Bohnert 1993, Hasegawa et al. 2000). Although some plants utilize ions for osmotic adjustment, others sequester Na^+ and other ions to keep them away from the metabolic sites (Blumwald 2000). Some halophytes grow better in soil containing elevated levels of NaCl by storing excessive amounts

Table 1. Summary of recent 'omics' studies related to drought, salinity and cold stresses in six genera of forest trees.

Family	Genus	Approach	Abiotic stress	Environment	References
Salicaceae	<i>Populus</i>	RNA-Seq-based transcriptome	Salinity tolerance	Greenhouse	Chen et al. (2012)
	<i>Populus</i>	RNA-Seq-based transcriptome	Drought tolerance	Greenhouse	Peng et al. (2012)
	<i>Populus</i>	RNA-Seq-based transcriptome	Salinity tolerance	Greenhouse	Jiang et al. (2012b)
	<i>Populus</i>	Transcriptome and gene expression analysis	Drought tolerance	Greenhouse	Pallara et al. (2012)
	<i>Populus</i>	Microarray-based transcriptome	Drought tolerance	Rain-shelter field	Yan et al. (2012)
	<i>Populus</i>	Microarray-based transcriptome	Salinity tolerance	Greenhouse	Beritognolo et al. (2011)
	<i>Populus</i>	Microarray-based transcriptome	Drought tolerance	Climate chamber	Raj et al. (2011)
	<i>Populus</i>	Proteome	Drought tolerance	Climate chamber	Durand et al. (2011)
	<i>Populus</i>	cDNA-AFLP-based transcriptome	Drought tolerance	Hydroponics greenhouse	Wang et al. (2011)
	<i>Populus</i>	Microarray-based transcriptome	Drought tolerance	Greenhouse	Berta et al. (2010)
	<i>Populus</i>	Microarray-based transcriptome	Salinity tolerance	Hydroponics greenhouse	Brinker et al. (2010)
	<i>Populus</i>	Gene expression	Drought tolerance	Greenhouse	Cocozza et al. (2010)
	<i>Populus</i>	Microarray-based transcriptome	Drought tolerance	Greenhouse	Cohen et al. (2010)
	<i>Populus</i>	Microarray-based transcriptome	Drought tolerance	Climate chamber	Hamanishi et al. (2010)
	<i>Populus</i>	Proteome	Drought tolerance	Greenhouse	Pechanova et al. (2010)
	<i>Populus</i>	Proteome	Drought tolerance	Greenhouse	Yang et al. (2010)
	<i>Populus</i>	Proteome	Drought tolerance	Greenhouse	Zhang et al. (2010)
	<i>Populus</i>	Proteome	Drought tolerance	Field and greenhouse	Bonhomme et al. (2009a)
	<i>Populus</i>	Proteome	Drought tolerance	Field	Bonhomme et al. (2009b)
	<i>Populus</i>	Gene and protein expression	Drought tolerance	Greenhouse	Regier et al. (2009)
	<i>Populus</i>	Proteome	Drought tolerance	Greenhouse	X.W. Xiao et al. (2009)
	<i>Populus</i>	Microarray-based transcriptome	Drought tolerance	Climate glasshouse	Wilkins et al. (2009)
	<i>Populus</i>	Proteome	Drought tolerance	Greenhouse	He et al. (2008)
<i>Populus</i>	Gene expression	Drought, salinity and cold tolerance	Greenhouse, climate chamber	Zhang et al. (2008)	
<i>Populus</i>	Transcriptome and proteome	Drought tolerance	Greenhouse	Bogeat-Triboulot et al. (2007)	
<i>Populus</i>	Transcriptome and proteome	Drought tolerance	Greenhouse	Plomion et al. (2006)	
<i>Populus</i>	Microarray-based transcriptome	Drought tolerance	Climate chamber	Street et al. (2006)	
<i>Populus</i>	Transcriptome and metabolome	Salinity and drought tolerance	Natural habitat	Brosché et al. (2005)	
<i>Populus</i>	Microarray-based transcriptome and suppression subtractive hybridization	Salinity tolerance	Hydroponics greenhouse	Gu et al. (2004)	
Pinaceae	<i>Pinus</i>	Microarray-based transcriptome	Drought tolerance	Greenhouse	Lorenz et al. (2011)
	<i>Pinus</i>	Proteome	Drought tolerance	Greenhouse	He et al. (2007)
	<i>Pinus</i>	Microarray-based transcriptome	Drought tolerance	Greenhouse	Lorenz et al. (2005)
	<i>Pinus</i>	Microarray-based transcriptome	Drought tolerance	Greenhouse	Watkinson et al. (2003)
	<i>Pinus</i>	cDNA-AFLP-based transcriptome	Drought tolerance	Hydroponics greenhouse	Dubos and Plomion (2003)
	<i>Pinus</i>	cDNA-AFLP-based transcriptome	Drought tolerance	Hydroponics greenhouse	Dubos et al. (2003)
	<i>Pinus</i>	Microarray-based transcriptome	Drought tolerance	Field	Heath et al. (2002)
	<i>Pinus</i>	Microarray-based transcriptome	Cold acclimation	Field, greenhouse, climate chamber	Joosen et al. (2006)
	<i>Picea</i>	Microarray-based transcriptome and metabolome	Cold acclimation	Field	Greene et al. (2012)
	<i>Picea</i>	Microarray-based transcriptome	Cold acclimation	Field	Holliday et al. (2008)
Myrtaceae	<i>Eucalyptus</i>	Gene expression	Drought tolerance	Climate chamber	Fossdal et al. (2007)
	<i>Eucalyptus</i>	Proteome	Drought tolerance	Field	Bedon et al. (2012)
	<i>Eucalyptus</i>	Metabolome	Drought tolerance	Greenhouse	Warren et al. (2012)
	<i>Eucalyptus</i>	RNA-Seq-based transcriptome	Drought tolerance	Field	Villar et al. (2011)
	<i>Eucalyptus</i>	Metabolome	Drought tolerance	Field	Arndt et al. (2008)
Fagaceae	<i>Quercus</i>	Microarray-based transcriptome	Drought tolerance	Greenhouse	Spieb et al. (2012)
	<i>Quercus</i>	Proteome	Drought tolerance	Greenhouse	Sergeant et al. (2011)
	<i>Quercus</i>	Proteome	Drought tolerance	Greenhouse	Echevarría-Zomeño et al. (2009)
	<i>Catsanea</i>	Gene expression	Cold acclimation	Field	Ibañez et al. (2008)

of Na⁺ in their cellular vacuoles (Thomas and Bohnert 1993, Tester and Davenport 2003, Flowers 2004). Striking differences in salinity tolerance exist among salt-susceptible and -tolerant species of poplar (Fung et al. 1998), with *Populus euphratica* (Oliv.) being an example of the latter (Brosché et al. 2005). Key salt-tolerance mechanisms identified in this species include compartmentalization of Cl⁻ in the vacuoles of root cortex cells, diminished xylem loading of NaCl, Na⁺ extrusion into the soil solution, while simultaneously avoiding excessive K⁺ loss by depolarization–activation of cation channels. Additionally, *P. euphratica* develops leaf succulence after prolonged salt exposure, resulting in salt dilution (Chen and Polle 2010). Intra-species comparative studies may provide additional information regarding salt-tolerance mechanisms in poplar. For example, white poplar (*Populus alba* [L.]) has considerable genetic variation for salt tolerance. Because this variation resulted from natural selection in contrasting ecological habitats, recent studies involving two *P. alba* genotypes—a salt-tolerant genotype of southern origin and a salt-sensitive genotype from the north of Italy—have shown substantial phenotypic and molecular differences in their responses to soil salinity (Beritognolo et al. 2007, Abbruzzese et al. 2009, Beritognolo et al. 2011).

Cold stress

Tree death from cold stress is expected to accelerate in coming decades as a result of climate change (e.g., extreme cold winter and harsh spring), and forest die-off events are already occurring in North America and Euroasia (Li et al. 2012b, Zhang et al. 2012). To survive cold winter conditions, temperate forest trees typically stop growing and develop cold hardiness prior to the onset of winter. In response to short days and low temperatures, metabolism shifts toward the production of storage compounds, such as vegetative storage proteins (Clausen and Apel 1991), and other biochemical and physical changes are initiated that allow plants to withstand low temperatures (Weiser 1970, Welling et al. 2002). Membranes are common sites of freezing injury, and cold-acclimation processes stabilize membrane structures to prevent their damage (Steponkus 1984, Palta 1990). One of the major steps taken by plants during the acquisition of freezing tolerance is to modify the saturation level of the fatty-acid side chains in membrane lipids to maintain membrane fluidity (Kodama et al. 1995, Falcone et al. 2004).

Sensing of abiotic stress and downstream signaling pathways

A better understanding of the mechanisms by which trees perceive environmental cues and transmit signals to activate adaptive responses is of fundamental importance in stress-biology research. Knowledge about stress-signal transduction is also

crucial for breeding and engineering tree tolerance to abiotic stress. Although they may be related in part, drought, salt and cold each cause a quite different response (Xiong et al. 2002). For example, cold may immediately result in mechanical constraints, changes in activities of macromolecules and reduced cell osmotic potential. Salinity includes both ionic and osmotic components. Given the multiplicity of stress signals, many different sensors are expected (Xiong et al. 2002). Recently, Park et al. (2009) identified a protein, pyrabactin resistant 1 (PYR1), which is involved in abscisic acid (ABA) signaling in *Arabidopsis thaliana* (L.) Heynh. Both PYR1 and PYR1-like receptors are necessary for many plant responses to ABA. Simultaneously, the ABA receptor RACR1, which belongs to the same PYR/PYL family of receptors, has also been identified (Ma et al. 2009). This family of receptor proteins appears to be highly conserved across crop plants, and recent work is aimed at elucidating equivalents in tree species (reviewed by Klingler et al. 2010).

Although the precise mechanism underlying stress perception is not well understood, there are several hypotheses related to how roots sense water deficiency. Abscisic acid accumulates in the soil solution, in response to decreased soil water, and may be involved in the sensing mechanism by which roots respond to a reduction in soil water availability (Slovik et al. 1995, Hartung et al. 1996). In addition, drought may be perceived through a reduction in cellular turgor pressure. Indeed, Urao et al. (1999) identified an *A. thaliana* trans-membrane histidine kinase, AtHK1, which may function as an osmosensor. AtHK1 senses osmotic changes and transmits a stress signal to downstream mitogen-activated protein kinase signaling cascades, which, in turn, induces drought-responsive gene expression (Urao et al. 1999). Importantly, two HKT1 homologs that can sense changes in solute concentration, similar to AtHK1, have been identified in *Eucalyptus camaldulensis* (Dehnh.) by Liu et al. (2001). ECHKT1 proteins may prove to be candidates when trees are bred for the perception of water stress and drought response signaling.

Downstream of water-deficit sensing, ABA is undoubtedly the plant hormone most intimately involved in stress-signal transduction. It is thought to play important roles in root-to-shoot signaling (Walton et al. 1976, Zeevaart and Creelman 1988, Davies and Zhang 1991) in the regulation of stomatal movement in response to drought (reviewed by Wilkinson and Davies 2002, Belin et al. 2010, Popko et al. 2010); and in the maintenance of root growth under mild-to-moderate drought stress, while restricting leaf growth (Hsiao and Xu 2000). In response to drought, growth allocation patterns in plants are altered and the variable growth rates observed in roots and shoots are shown to be correlated with ABA levels, but this regulation of growth may be modulated by ethylene (Sharp et al. 2004).

Although calcium sensing is known to be mediated by a family of calcineurin B-like proteins (CBLs) interacting with protein

kinases (Luan et al. 2002), our knowledge of this pathway in forest trees is scant. Among different members of the CBL family in *P. euphratica*, *PeCBL2*, *PeCBL3* and *PeCBL5* reached maximum transcript levels after 3 h and *PeCBL1*, *PeCBL4* and *PeCBL9* after 12 h of salinity stress (Zhang et al. 2008). Transcription of *PeCBL10* decreased after a 24-h salt shock, as well as after long-term salt acclimation, suggesting its role as a negative regulator of salt adaptation in *P. euphratica* (Ottow et al. 2005). In addition, it has been shown that salinity caused a significant increase in leaf Ca²⁺ sensor calmodulin in poplar species after the onset of stress. This response was most pronounced in salt-tolerant *P. euphratica*, suggesting that this species is more sensitive in sensing soil salinity than salt-susceptible poplar species (Chang et al. 2006).

Molecular outputs of stress-responsive genes

Water deficit

The accumulation of the hydrophobic late-embryogenesis-abundant (LEA) proteins is an important aspect of environmental stress in plants that is commonly associated with tolerance to water-deficit conditions (Welin et al. 1994). Recent evidence suggests that LEA proteins may have an important role in the stabilization of other proteins and membranes, as well as the prevention of protein aggregation during periods of water deficit (Close 1996, Goyal et al. 2005). For example, a rapid induction of dehydrins, LEA family proteins, was observed in response to osmotic stress in poplar (Caruso et al. 2002). Similar increases in transcript or protein levels of LEA family members have also been observed in Norway spruce (Blodner et al. 2007).

Aquaporins play a key role in drought stress. They are components of water channels found in cellular membranes that are primarily responsible for water flux, and are crucial for maintaining proper water balance (reviewed by Maurel et al. 2008). Two other classes of water-transport proteins, plasma membrane intrinsic proteins (PIPs) and tonoplast membrane intrinsic proteins, are vital for maintaining water status in the plant, which is fundamental for photosynthesis, and thus for growth. In *Eucalyptus* spp., a reduction in PIPs resulted in a suppression of growth (Tsuchihira et al. 2010). In white poplar, Berta et al. (2010) identified five transcripts coding for aquaporin membrane proteins that were up-regulated following re-watering in trees that experienced drought stress. Accumulation of aquaporins following re-watering may be integral to restoration of water transport of plants under well-watered conditions (Hamanishi and Campbell 2011). It has been recently shown that members of the PIP1 family of aquaporins are important for recovery from xylem embolisms in *P. trichocarpa* (Secchi and Zwieniecki 2010).

A deeper understanding of drought tolerance in forest trees has been achieved through the study of the stomatal development pathway. Hamanishi et al. (2012) used *Populus balsamifera* (L.) to investigate the impact of drought on

stomatal development during leaf formation. *Populus* homologs of *STOMAGEN*, *ERECTA* (*ER*), *STOMATA DENSITY AND DISTRIBUTION 1* (*SDD1*) and *FAMA* showed variable transcript abundance patterns congruent with their role in the modulation of stomatal development in response to drought. Conversely, there was no significant variation in transcript abundance between genotypes or in response to water stress for *YODA* (*YDA*) and *TOO MANY MOUTHS* (*TMM*). These findings highlight the potential for limiting water loss from leaves by altering stomatal development during leaf expansion (Hamanishi et al. 2012). However, further studies are needed to assess the long-term effects of modulating stomatal development. Moreover, receptor-like kinases (RLKs) have been implicated in plant responses to several types of abiotic stress, including drought (Marshall et al. 2012). Berta et al. (2010) showed that short periods of water deficit-induced expression of a specific *RLK* gene in wood-forming tissues of poplar. In addition, water-use efficiency (WUE) was improved by the expression of a poplar ortholog of *ER*, *PdERECTA*, cloned from (*Populus nigra* [L.] × (*Populus deltoides* [W. Bartram ex Humphry Marshall] × *P. nigra*)), in *A. thaliana* (Xing et al. 2011). Although the biochemical basis for this effect of *PdERECTA* is still unknown, the reduction in stomatal density controlled by *ER* is expected to contribute to a decreased transpiration rate and improved WUE (Xing et al. 2011).

Salinity stress

Among genes up-regulated by salt stress in *P. euphratica* were those involved in ionic and osmotic homeostasis. Gu et al. (2004) showed that the most highly up-regulated transcripts corresponded to a heat-shock protein (Hsp90). The GRAS/SCL-type transcription factor gene *PeSCL7* was isolated from *P. euphratica*, and it was shown to be induced during the early stages of severe salt stress. Over-expression of *PeSCL7* in transgenic *A. thaliana* led to plants with improved tolerance to drought and salt stresses (Ma et al. 2010). In Italian *P. alba* clones varying in salt tolerance, the transcriptional profiles of tonoplast and plasma membrane H⁺-ATPase genes revealed different regulatory patterns in response to salinity (Beritognolo et al. 2007). In the relatively salt-tolerant genotypes, tonoplast-ATPase genes were up-regulated after intermediate (2 weeks) and down-regulated after prolonged (4 weeks) exposure to high salinity. In contrast, the salt-susceptible genotype showed no regulation in transcript levels of both plasma membrane-ATPases and tonoplast-ATPases, indicating that this genotype lacks a sensitive signal-transduction network, which is essential to trigger an active response to salt stress (Beritognolo et al. 2007).

Cold stress

The best characterized cold-response pathway in model plants is the C-repeat-binding factor/dehydration-responsive element binding (CBF/DREB1) pathway (Thomashow 1999,

Yamaguchi-Shinozaki and Shinozaki 2006). The CBF pathway is also operational in trees (Puhakainen et al. 2004), and CBF orthologs have been described in various woody plants, including poplar (Benedict et al. 2006) and *Eucalyptus gunnii* (Hook.f.) (El Kayal et al. 2006). Interestingly, while some poplar CBF genes were up-regulated in leaves by cold, others were up-regulated in perennial tissues (Benedict et al. 2006), suggesting that specific CBFs might have distinct roles in perennial (stem) and annual (leaf) tissues. Other findings confirm this conclusion and relate to the poplar homologs of *A. thaliana* CBF-regulon genes (e.g., *CBF1*, *CBF3* and Inducer of *CBF3* Expression 1 (*ICE1*)) that were up-regulated as much as 391-fold in the winter (dormancy) stems (Ko et al. 2011). *ICE1* is a MYC-like basic helix–loop–helix transcription factor that activates *CBF3* and *COR* genes in response to low temperatures in *A. thaliana* (Chinnusamy et al. 2003).

Dehydrins also play an important role in cold-stress tolerance in trees during overwintering. Cold-inducible dehydrin genes in trees contain CRT elements in their promoters (Puhakainen et al. 2004, Benedict et al. 2006, Wisniewski et al. 2006), suggesting that CBFs control their expression at low temperatures. Expression analysis of four CBF genes (*CBF1a*, *CBF1b*, *CBF1c* and *CBF1d*) from *E. gunnii* revealed that they are all preferentially induced by cold, except for the *CBF1c* gene, which is more responsive to salt (Navarro et al. 2009). The high accumulation of the CBF transcript, observed in response to different types of cold treatment, might be key to winter survival of this evergreen, broad-leaved tree (Navarro et al. 2009). Four CBF transcription factors were identified in the cold-acclimation process in birch (*Betula pendula* [Roth]) and designated *BpCBF1* to -4 (Welling and Palva 2008). Ectopic expression of birch CBFs in *A. thaliana* resulted in constitutive expression of endogenous CBF target genes and increased freezing tolerance of non-acclimated transgenic plants, suggesting that, in addition to their role in cold acclimation during the growing season, CBFs appear to contribute to control of winter hardiness.

Epigenetic responses to abiotic stress

It is now generally accepted that epigenetic changes are likely caused by either genomic stresses or environmental stresses, or both (Bonasio et al. 2010). At the molecular level, epigenetic phenomena are mediated by reversible DNA methylation and histone modifications, including acetylation, methylation, ubiquitylation, phosphorylation and sumoylation, and by small ribonucleic acids (RNAs) that can alter regulatory states of genomic regions, as discussed below. So far, only a few aspects of epigenetic regulation of abiotic stress response have been documented in plants, but results from recent research are helping to identify the epigenetic factors that mediate responses to abiotic stresses (Chinnusamy et al. 2007,

Hollick 2008, Chinnusamy and Zhu 2009). It is likely that epigenetic variations contribute to plant potential to adapt to stress(es) and, like genetic diversity, are under environmental selection (Gutzat and Scheid 2012).

Yakovlev et al. (2012) recently suggested the existence of epigenetic memory in Norway spruce. This phenomenon is fixed by the time the seed is fully developed, is long-lasting and affects the growth cycle of these trees, allowing them to adapt rapidly to changing environments. Thus, the epigenetic memory effect has practical implications for tree breeding and seed production, and care should be taken to ensure that family seed lots generated for progeny testing and for selection of the next generation are produced under similar temperature and day-length conditions. A recent review considers the role of epigenetics as a new source of adaptive traits in forest-tree breeding, biotechnology and ecosystem conservation in response to climate change (Bräutigam et al. 2013). Epigenetic changes might contribute not only to the phenotypic plasticity and adaptive capacity of forest trees, but also to their ability to persist in variable environments.

Another recent study highlights the importance of epigenetic mechanisms related to the adaptation of long-lived species to local environments (Raj et al. 2011). The authors indicate that clonally propagated poplar genotypes grown under drought stress showed the most pronounced location-specific patterns in transcriptome response and genome-wide DNA methylation. Analysis of DNA methylation and traits related to biomass productivity in hybrid poplar (*P. deltoides* × *P. nigra*) (Gourcilleau et al. 2010, Hamanishi and Campbell 2011) indicated a positive correlation between these variables under well-watered conditions. However, genotypes that exhibited reduced growth under water-deficit conditions showed the greatest variation in DNA methylation, demonstrating that DNA methylation could be responsible for refining gene expression in poplar during water stress. These findings expose a variety of molecular mechanisms available to defend against abiotic stress, thus harnessing epigenetics for forest-tree management and improvement.

Small RNA in abiotic stress

Genetic and genomic studies in a variety of forest-tree species have begun to unravel the complexity of stress-response mechanisms, but the focus has been on stress-responsive protein-coding genes. It is now clear that small RNAs play an important role in plant stress responses, but a detailed understanding of the molecular events that regulate gene expression and give rise to abiotic stress tolerance is still lacking. Initial clues that small RNAs are involved in plant stress responses stem from studies of post-transcriptional regulation of gene expression (reviewed by Covarrubias and Reyes 2010).

Small RNAs do not encode proteins; they alter the expression of mRNAs by cleavage or repression of translation, or by methylation of DNA (Ramachandran and Chen 2008). Several groups of small RNAs have been described: microRNAs (miRNAs), small-interfering RNAs, tiny non-coding RNAs and small modulatory RNA. MicroRNAs are single-stranded RNAs, 19–25 nucleotides in length, that are generated from endogenous hairpin-shaped transcripts (Ambros et al. 2003, Cullen 2004). They play key roles in an array of regulatory pathways involved in abiotic stress responses, such as salinity (Jia et al. 2009, Li et al. 2011, 2013, Ren et al. 2012), drought (Zhao et al. 2007, Covarrubias and Reyes 2010, Li et al. 2011, Shuai et al. 2013) and cold (Zhou et al. 2008, Chen et al. 2012). MicroRNAs serve as guide molecules by pairing with their mRNA targets, leading to cleavage or translational repression. Several stress-responsive miRNAs have been discovered in trees, especially in poplar, a model organism that is often used to characterize functionality in forest trees. An early example was the discovery of novel stress-responsive miRNAs in *P. trichocarpa* that target developmental and stress- and defense-related genes (Lu et al. 2005). In addition, several differentially regulated miRNAs have also been identified in salt-stressed trees. In *P. trichocarpa*, miR530a, miR1445, miR1446a-e, miR1447 and miR1711-n were down-regulated, whereas miR482.2 and miR1450 were up-regulated in response to salt stress (Lu et al. 2008). Subsequently, miR398 was reported to be induced by salt stress in *Populus tremula* (L.) (Jia et al. 2009). Recently, the small RNAome, degradome, and transcriptome were investigated in *P. euphratica*, which led to the identification of a large number of new miRNAs that are responsive to salt stress. Degradome sequencing has been used to identify miRNA cleavage sites; once target genes were validated, their expression was verified by transcriptome sequencing. Interestingly, expression of 15 miRNA-target pairs displayed pattern reversal under salt stress (Li et al. 2013). Moreover, three novel miRNAs and nine conserved miRNAs from *P. trichocarpa* that are responsive to drought were recently discovered by high-throughput sequencing, and their targets were identified using degradome sequencing (Shuai et al. 2013). Comparing these miRNAs with those identified in other plant species led to the discovery that an *A. thaliana* homolog of Ptc-miR159 targets a MYB transcription factor. The ABA-induced accumulation of the miR159 homolog leads to MYB transcript degradation, desensitizing hormone signaling during seedling stress responses (Reyes and Chua 2007). Ptc-miR159 was confirmed to target a methionine sulfoxide reductase (MSR), an enzyme involved in regulating the accumulation of reactive oxygen species, which can damage proteins in plant cells (Romero et al. 2004). Regulation of the MSR gene by Ptc-miR159 may occur through a homeostatic mechanism in response to drought stress in *P. trichocarpa* (Shuai et al. 2013). Ptc-miR473 is up-regulated in *P. trichocarpa* in response to drought, and it targets Vein Patterning 1 (VEP1),

which belongs to a short-chain dehydrogenase/reductase superfamily (Herl et al. 2009). In addition to VEP1, this miRNA regulates the expression of the GRAS protein, both of which are responsive to drought stress, suggesting that this may be a major drought-tolerance mechanism in *P. trichocarpa*.

The expression of miRNAs in response to cold stress has also been examined in poplar. Lu et al. (2008) identified 19 cold-responsive miRNAs in *P. trichocarpa*. Of these, they showed that miR397, miR169, miR168a,b and miR477a,b are up-regulated, while miR156g-j, miR475a,b and miR476a are down-regulated in response to low temperatures. Because the genes predicted to be targets of these miRNAs have such diverse functions, it is thought that these miRNAs play important regulatory roles in various aspects of the plant's response to cold stress. Using deep sequencing, Chen et al. (2012) have identified other stress-responsive miRNAs in *Populus beijingensis* (W.Y. Hsu).

Although these findings support a role of miRNA in abiotic stress tolerance, they are thought to be involved at many different levels, only a few of which have been documented so far. Additional deep sequencing and miRNA precursor expression studies will identify sequence variations, including nucleotide sequence polymorphisms and length variation in mature miRNAs, which may provide new insights into how these agriculturally important phenotypes can be regulated.

Genomics-assisted QTL studies

Quantitative-trait loci mapping has been an attractive forward-genomics approach to elucidating complex traits. In forest trees, it has been applied to traits such as drought, salinity and cold stresses (Table 2); however, it has not been able to reveal the specific underlying genes as it has in model systems or a few major crop species (Neale and Kremer 2011). Frequently, innate genetic variation is studied via mapping QTLs; however, considering transcripts to be an individual phenotype has led to the emerging field of expression QTL (eQTL) analysis (Kliebenstein 2009). The use of specific eQTLs has helped elucidate the molecular basis of some quantitative traits. Accordingly, transcriptomics has become a powerful tool for candidate-gene discovery. Applying gene-expression profiling, QTL mapping and phenotyping to segregating populations will help to identify the drivers of pathways or factors responsible for apparent variation. In forest trees, these technologies can provide unique insights into the genetic architecture underlying abiotic stress tolerance that would not be possible via traditional, single-gene approaches. In an early genomics study of drought stress in poplar, QTL analysis was used to show that a number of genes with differential expression between the two extreme genotypes of an F₂ mapping population co-located to specific genomic regions. These were known to be highly divergent for many phenotypic traits and may provide clues as to the genetic mechanisms by which species adapt to drought

Table 2. Summary of recent QTL and expression-QTL studies related to drought, salinity and cold stress in seven genera of forest trees.

Family	Genus	Cross	Mapping population	Approach	Abiotic stress	Environment	References
Salicaceae	<i>Populus</i>	<i>P. deltoides</i> × <i>P. trichocarpa</i>	F1	QTL	Drought tolerance	Field	Monclus et al. (2012)
	<i>Populus</i>	<i>P. deltoides</i> × <i>P. nigra</i>	F1	QTL	Drought tolerance	Field	Dillen et al. (2008)
	<i>Populus</i>	<i>P. trichocarpa</i> × <i>P. deltoides</i>	F2	Expression-QTL	Drought stress	Greenhouse	Street et al. (2006)
	<i>Populus</i>	<i>P. trichocarpa</i> × <i>P. deltoides</i>	F2	QTL	Drought stress	Greenhouse	Street et al. (2006)
	<i>Populus</i>	<i>P. trichocarpa</i> × <i>P. deltoides</i>	F2	QTL	Osmotic potential	Field	Tschaplinski et al. (2006)
	<i>Salix</i>	<i>S. dasyclados</i> × <i>S. viminalis</i>	F2	QTL	Freezing tolerance	Field	Tsarouhas et al. (2004)
	<i>Salix</i>	<i>S. dasyclados</i> × <i>S. viminalis</i>	F2	QTL	Drought tolerance	Greenhouse	Rönberg-Wästljung et al. (2005)
Pinaceae	<i>Pinus</i>	<i>P. pinaster</i>	F1	QTL	Drought tolerance	Field	Brendel et al. (2002)
	<i>Pseudotsuga</i>	<i>P. menziesii</i>	F1	QTL	Cold hardiness	Field	Wheeler et al. (2005)
	<i>Pseudotsuga</i>	<i>P. menziesii</i>	F2	QTL	Cold hardiness	Field	Jermstad et al. (2001)
Myrtaceae	<i>Eucalyptus</i>	<i>E. grandis</i> × <i>E. urophylla</i>	F1	QTL	Drought tolerance	Greenhouse	Teixeira et al. (2011)
	<i>Eucalyptus</i>	<i>E. nitens</i>	F1	QTL	Frost tolerance	Greenhouse	Byrne et al. (1997)
Fagaceae	<i>Quercus</i>	<i>Q. robur</i> × <i>Q. robur</i> ssp.	F1	QTL	Drought tolerance	Field	Brendel et al. (2008)
	<i>Quercus</i>	<i>Q. robur</i> × <i>Q. robur</i> ssp.	F1	QTL	Drought tolerance	Greenhouse	Gailing et al. (2008)
	<i>Quercus</i>	<i>Q. robur</i> × <i>Q. robur</i> ssp.	F1	QTL	Drought tolerance	Field	Parelle et al. (2007)
	<i>Castanea</i>	<i>C. sativa</i>	F1	QTL	Drought tolerance	Field	Casasoli et al. (2006)

(Street et al. 2006). With the advent of high-throughput omics technologies, interest has been renewed in eQTL mapping for biomass production and response to drought stress, especially for *Populus*, where screening of entire populations for association of gene expression with targeted traits is more feasible than with other tree species. Establishing a causal relationship between genotypes and phenotypes is fundamental to our understanding of the genetic basis of abiotic stress traits. Consequently, it is important for breeding programs, particularly those for biomass production and drought tolerance, the traits for which breeders have the greatest hope when it comes to marker-assisted selection (MAS).

Genomic selection

Genomic selection has been recently proposed as an alternative to MAS in crop improvement (Bernardo and Yu 2007, Heffner et al. 2009). It is a form of MAS in which markers spanning the entire genome are used such that all QTLs are in linkage disequilibrium with at least one marker (Goddard and Hayes 2007). With forest trees, GS is extremely appealing due to the prospects of improving accuracy when selecting for traits with low heritability (e.g., biomass productivity and abiotic

stress tolerance) and where long generation times and late-expressing, complex traits are involved (Grattapaglia and Resende 2010).

A few studies have evaluated the prospect of GS for forest-tree species, and concluded that GS can be superior to phenotype-based selection with respect to time. For example, Grattapaglia and Resende (2010), using deterministic models, analyzed the use of GS in tree improvement and demonstrated its great potential in accelerating traditional breeding programs. This was confirmed by a simulation study using *Cryptomeria japonica* [(L.f.) D. Don] (Iwata et al. 2011). Similarly, Denis and Bouvet (2013) demonstrated how GS efficiency in *Eucalyptus* is augmented by increasing the relationship between the training and candidate populations, the training population size and heritability. In addition, the first such study with *Pinus taeda* demonstrated the value of GS, provided that the models were used at the relevant selection age and within the breeding zone where marker effects were estimated (M.F.R. Resende et al. 2012b). Likewise, the first experimental results with GS in *Eucalyptus* demonstrated its value in understanding the quantitative-trait variation in forest trees and its power as a tool for applied tree breeding (M.D. Resende et al. 2012). These studies indicate that GS can be an effective strategy for selecting

among genotypes whose phenotypes are not yet apparent. Denser markers will become available soon, and this may further improve the ability of GS to predict genetic values in forest trees.

However, successful application of GS in tree breeding programs aimed at developing trees that are tolerant to drought, salinity and cold stresses will require comprehensive physiological information that relies on rigorous phenotyping, a crucial step before implementing the genetic strategies to elucidate the complex multi-layered abiotic stress-tolerance mechanisms, and to further investigate molecular-breeding approaches for tree improvement. Accurate and precise phenotyping strategies are also necessary to empower high-resolution linkage mapping and genome-wide association studies and for training GS models in plant improvement (Cobb et al. 2013). In forest trees, this will require the utilization of sophisticated, non-destructive multi-spectral imaging techniques. For example, near-infrared spectroscopy, canopy spectral reflectance and infrared thermography can be used to assess biomass productivity and plant water status, and to detect abiotic stresses at the individual-tree level. These field-based, rapid phenotyping tools can be mounted on an unmanned aerial vehicle (UAV), commonly known as a drone, which can be directed with a global positioning system to enhance the precision and accuracy of phenotyping under field conditions, and without the need for destructive sampling.

These high-throughput methods create opportunities to generate comprehensive phenotypic information, such as abiotic stress tolerance and biomass estimation and, thus, to uncover phenotype-to-genotype relationships and their relevance for improving tolerance to abiotic stresses. Once validated, single trees harboring the gene or QTL of interest will be useful for dissecting the molecular mechanism(s) involved in the desired trait(s), and will also be of immediate value as elite breeding material(s).

As applications of GS have captured the interest of the global forest-tree breeding community, in both the public and private sectors, there are now several large translational tree genomics projects around the world aimed at bringing GS to applications in commercial breeding programs.

Strategies for genetic engineering

The challenge for scientists and breeders alike is not only to understand the molecular basis for complex-trait variation, but also to use that knowledge to develop trees that can be grown under sub-optimal conditions (e.g., on marginal land), where resources (e.g., water) are limiting.

The discovery of stress-tolerance genes and manipulating their expression will become increasingly important to maintain productivity and ensure sustainability. Several genes involved in plant responses to drought, salt or cold stress have been previously studied at the transcriptional level. The products of

these stress-inducible genes have been classified into two groups: (i) those that directly protect against environmental abiotic stresses; and (ii) those that regulate gene expression and signal transduction in the stress response. Although candidate genes have been identified in a number of food crops, here we focus on the use of transgenesis to develop those traits in forest trees.

Transgenes to improve tolerance to drought and salt stress

Recent studies of molecular networks concerned with stress sensing, signal transduction and stress responses in plants have revealed that salinity and water stress are intimately related. Engineering tolerance to drought and salinity stress using some of the genes involved in these mechanisms may simultaneously improve drought tolerance and WUE. In this section, we survey the various strategies that have been used to produce transgenic trees having improved tolerance to drought and salinity stress. We also discuss criteria for choosing which genes to focus on, what promoters to utilize and transgene sources for successfully engineering trees that can resist far greater levels of stress than their non-transgenic counterparts.

Zhang et al. (2013) recently showed that constitutive expression of the wintersweet (*Chimonanthus praecox* [(L.) Link]) fatty acyl–acyl carrier protein thioesterase (*CpFATB*) in poplar activates an oxidative signal cascade and leads to drought tolerance. Their transgenic poplars maintained significantly higher photosynthetic rates, suggesting that changes in fatty-acid composition and saturation levels may be involved in tolerance to dehydration. Another study reported that over-expression of several resistance genes in poplar enhances tolerance to environmental stressors. The genes included: *vgb*, which encodes a bacterial hemoglobin; *SacB*, encoding an enzyme involved in fructan biosynthesis in *Bacillus subtilis*; and *JERF36*, a tomato (*Solanum lycopersicum* [L.]) gene encoding jasmonate/ethylene-responsive factor protein. The improved growth seen in the transgenics can be primarily attributed to higher WUE and fructan levels, and better root architecture under drought and salinity stress. Gene stacking such as this has led to the discovery that multiple genes from various pathways converged to improve stress tolerance that could not be achieved by a single-gene approach (Su et al. 2011). However, to better understand cross-talk among genes, comparing poplars transformed with several genes with those with a single gene may help to identify the relationships between traits and gene function.

A recent study showed that over-expression of the GSK3/shaggy-like kinase (*AtGSK1*) gene from *A. thaliana* conferred drought and salt tolerance in poplar (Han et al. 2013). The transgenic poplars showed higher rates of photosynthesis, stomatal conductance and evaporation under stress. Dehydrins are Group II, LEA proteins that putatively act as chaperones in

stressed plants. Over-expression of the SK2-type dehydrin gene from *P. euphratica* in a *P. tremula* × *P. alba* increased drought tolerance. Presumably dehydrin expression protected the cell membranes and/or macromolecules in transgenic lines from the shortage of water, resulting in enhanced water conservation and reduced water loss under drought stress (Wang et al. 2011).

Recent developments highlight the importance of halophytes in understanding salinity tolerance mechanisms in forest trees. For example, transgenic poplars expressing a manganese superoxide dismutase gene from *Tamarix androssowii* (L.) exhibited enhanced salt tolerance (Wang et al. 2010). In contrast, ectopic expression of the tomato jasmonic ethylene-responsive factor gene in poplar led to increased salt tolerance (Li et al. 2009). Transgenic plants grew vigorously when exposed to high salt in both the greenhouse and the field. Moreover, in the absence of salt, the transgenics grew significantly taller than the non-transgenic controls.

When Takabe et al. (2008) over-expressed the DnaK molecular chaperone from a halotolerant cyanobacterium *Aphanothece halophytica* (Fremy) (ApDnaK) in *P. alba*, the growth rate of transgenic plants was similar to that of wild-type (WT) plants under low light. However, under high light, the transgenics grew faster and had higher cellulose content. Transgenic plants also recovered more rapidly from high salinity, drought and low temperature than WT plants when grown under low light. More recently, when Jiang et al. (2012a) over-expressed a vacuolar Na⁺/H⁺ antiporter gene from *A. thaliana*, *AtNHX1*, in poplar (*Populus* × *euramericana*), the transgenics were more resistant to NaCl than WT plants. The transgenic plants also grew faster, had greater photosynthetic capacity and accumulated more Na⁺ and K⁺ in their roots and leaves than WT plants.

Transgenes to improve tolerance to cold stress

Cold is an important environmental factor that can significantly limit the growth and distribution of trees. Exposure to low temperatures during the growing season is a leading cause of stress and damage to tree seedlings (Blennow and Lindkvist 2000, Li et al. 2002). Short days and cold temperatures trigger various changes that allow plants to survive low temperatures (Welling et al. 2002). Forest trees are also susceptible to freezing damage during the winter, while the plants are dormant, because they are not sufficiently cold-hardy (Lund and Livingston 1999). Membranes must be stabilized to prevent injury (Steponkus 1984, Palta 1990), but membrane fluidity must be maintained at low temperatures (Beney and Gervais 2001). Thus, during cold acclimation, plants modify the saturation of the fatty-acid side chains in their membrane lipids (Kodama et al. 1995).

Plant responses to low temperatures are manifested at the physiological and molecular levels (Agarwal et al. 2006); a specific set of genes is activated and their products are involved in

the protection of cellular machinery from damage associated with the cold (Thomashow 1999, Shinozaki et al. 2003). For example, elevated levels of proline are shown to be associated with cold stress and its effect has been alleviated in transgenic hybrid larch (*Larix leptoeuropaea* [Dengler]) by over-expressing a *Vigna aconitifolia* gene for pyrroline 5-carboxylate synthase, which catalyzes the rate-limiting step in proline biosynthesis (Gleeson et al. 2005).

The C-repeat-binding factor (CBF) is a transcription factor that binds to a conserved *cis* element, and is involved in low-temperature signaling in *A. thaliana* (Jaglo-Ottosen et al. 1998). Over-expression of the *AtCBF1* gene from *A. thaliana* in hybrid aspen (*P. tremula* × *P. alba*) led to a significant increase in the tolerance of its non-acclimated leaves and stems to freezing (Benedict et al. 2006). Over-expression of a ERF/AP2 transcription factor encoded by the pepper (*Capsicum annuum* [L.]) *CaPF1* gene in eastern white pine (*Pinus strobus* [L.]) led to a dramatic increase in its tolerance to freezing, as well as the drought and salt stress mentioned above (Tang et al. 2007). The levels of putrescine, spermidine and spermine remained constant in *CaPF1*-over-expressed pine lines, whereas their levels decreased in the controls, suggesting that the improved tolerance was associated with polyamine biosynthesis.

The *ThCAP* gene from *Tamarix hispida* (Willd.) encodes a cold-acclimation protein. Over-expression of *ThCAP* in transgenic poplar (*Populus davidiana* [Dode] × *Populus bolleana*) led to greater resistance to low temperatures than seen with non-transgenic plants (Guo et al. 2009). Li et al. (2012a) reported that over-expressing two CBL genes from *P. euphratica* in white poplar confers cold-stress tolerance. Moreover, when two C-repeat-binding factor genes isolated from *E. gunnii*, EguCBF1a and -b, were constitutively expressed in a cold-sensitive *Eucalyptus* hybrid, it showed an improvement in freezing tolerance and development (Navarro et al. 2011). Additionally, a significant increase in freezing tolerance in hybrid poplar (*P. alba* × *Populus glandulosa* [Uyekii]) was achieved on a small scale following over-expression of the *Populus tomentosa* (Carriere) ω-3 fatty-acid desaturase gene, *FAD3* (Zhou et al. 2010).

These selected advances in engineering plant tolerance to abiotic stress confirmed the ability of stress-associated genes to improve drought, salinity and cold tolerance and will likely provide an approach to breed forest trees that can withstand climate change. Yet, clear-cut effects of stress-associated genes still need to be assessed over extended periods of time. Although over-expression of some genes may lead to dwarf phenotypes, as is the case in other plants with reduced yields, the use of stress-inducible (Kim et al. 2011, B.Z. Xiao et al. 2009), novel, moderately constitutive (Chen et al. 2013) or tissue-specific (Jeong et al. 2010) promoters may overcome this drawback.

Collectively, these results demonstrate the potential of genetic engineering for improving abiotic stress tolerance in forest trees.

Future directions in abiotic stress-tolerance research

Forest trees have evolved sophisticated systems for responding to abiotic stresses during their long life spans. Physiological and molecular analyses have allowed us to better understand their responses, and to determine which genes have the greatest impact under field conditions. Genome sequences have provided access to essential information on some genes (e.g., gene products and their function, transcript levels, putative *cis*-acting regulatory elements and alternative splicing patterns). Below, we offer our perspective on research directions for the next decade, in several topics.

Stress physiology

In order to fully assess abiotic stress responses at the whole-plant level, we need to gain better insight into tree stress physiology, especially water and ion transport, growth cycles in response to changing environments and the ability to recover from stress.

Genetic analysis

A more complete understanding of genes that can be used for forest-tree improvement has been hampered by their recalcitrance to genetic analyses. An interdisciplinary approach that combines genetics, genomics and phenomics is needed to better understand as-yet unexplored mechanisms of abiotic stress tolerance in forest trees and how they can be manipulated to overcome such stresses.

Systems-biology approaches

With recent technological advances in the areas of genomics, digital imaging and computational biology, we are now well poised to use systems-biology approaches to elucidate the molecular mechanisms of abiotic stress in forest trees at the whole-plant and -organ level. Integrating data collected at different levels into networks, in the context of the whole tree, may uncover complex molecular mechanisms and pathways that underpin the abiotic stress response. Ultimately, this holistic approach will deepen our understanding of stress responses and enhance our ability to develop desired tree phenotypes.

Next-generation RNA-sequencing

This technique can provide ready access to transcriptome profiling at an unprecedented level, thus replacing microarrays as the tool of choice for genome-wide transcriptome studies. RNA-sequencing can also provide new insights into the regulatory mechanisms underlying eQTL. Yet, improvements are still needed to overcome problems related to multireads and splice variants. Next-generation genomics will also facilitate our understanding of the genetic basis of variation in complex

phenotypic traits within natural populations. This opens the door to exploiting natural variation of forest-tree species.

Phenotyping

Overcoming the phenotyping bottleneck is crucial to understanding abiotic stress responses of forest trees in the field. Heterogeneous conditions, potential abiotic stress combinations and climate change will make it challenging to conduct field tests for abiotic stress tolerance in forest trees. An amalgam of approaches will likely be needed to significantly improve forest-tree performance in the field. It must also include careful attention to variation in field conditions, and extensive testing in both the greenhouse and the field. Of particular concern are field tests for genetically engineered trees; they must include, in addition to transgene confinement, stable transgene expression in a variety of environments, from year to year, and after trees have undergone the transition from juvenility to maturity. Relating sequence information to the phenotype of plants is critical for the identification and characterization of genes underlying abiotic stress traits. However, thanks to next-generation phenomic platforms, contributions to abiotic stress tolerance are being quantified with increasing ease. Unique and purpose-built phenotyping platforms, including field technologies (e.g., stationary or robot-mounted field sensors, and high-resolution, multi-spectral mapping from UAVs), are becoming available and can help alleviate two phenotyping impediments: (i) our capacity to more precisely and accurately quantify the development and functioning of plants than with traditional methods; and (ii) analysis of the tremendous volume of multi-dimensional bio-imaging data being collected. In the long term, this will allow automated measurement of a large suite of complex developmental and physiological traits in plantations. This, in turn, is critical to assigning biological functions to genes through forward or reverse genetics. It will also empower high-resolution linkage mapping and genome-wide association studies, and be used to train GS models for tree improvement.

Acknowledgments

The authors thank Torgny Näsholm for the invitation to write this review. They apologize to all their colleagues whose work was not described here due to space limitations.

Conflict of interest

None declared.

Funding

This research on abiotic stress was supported by the Brain Gain Program (Rientro dei cervelli) of the Italian Ministry of Education, University and Research (A.H.), and grants from the

European Community's Seventh Framework Program (WATBIO FP7-311929) to A.H.

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