Gene Activity Test Determines Cold Tolerance in Douglas-fir Seedlings

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Forest tree nurseries rely on a tight scheduling of operations to be able to deliver vigorous seedlings to the planting site. Cooler or freezer storage is often used to maintain planting stock in an inactive condition and to ensure a plant supply for geographically diverse planting sites, which is a requirement for large-scale or internationally operating nurseries.

Nursery growers often wish to know the earliest possible time when they can lift and store seedlings, and optimum scheduling depends on the physiological condition of the seedlings. Lifting and storage of insufficiently hardened plants reduces their vitality and may lead to cold damage, dehydration, and fungal infection. To prevent this kind of damage and its adverse economic effects on nurseries and end-users, it is of vital importance to be able to accurately determine the peak physiological condition for lifting or transfer. Cold hardiness testing is one method to determine seedling physiological condition because it is strongly linked to the seedling dormancy cycle and stress resistance, and is influenced by seed source, nursery practices, and environment (Faulconer 1988; Burr 1990).

Cold hardiness is traditionally defined as a minimum temperature at which a certain per-

centage of a random seedling population will survive or will sustain a given level of damage (Ritchie 1984). The term LT_{50} (lethal temperature for 50% of a population) is commonly used to define the hardiness level. Simpson (1990) found that LT_{50} at lifting correlated well with first-year survival and shoot growth of conifer seedlings. Over the past several decades, cold hardiness has been measured using the whole plant freeze test (Tanaka and others 1997), freeze induced electrolyte leakage (Burr and others 1990), or chlorophyll fluorescence (L'Hirondelle and others 2006). This paper describes a new technology that has emerged for cold hardiness testing.

New Technology

In 2006, a new method to measure cold hardiness was introduced by NSure, a spin-off company from Wageningen University in The Netherlands. The test is based on measuring the activity level of a carefully selected set of genes. When a gene becomes active, it produces a molecule called messenger RNA, or mRNA. This molecule travels from the nucleus to the cytoplasm and triggers subsequent actions of the cell. The NSure test measures the relative amount of certain mRNA molecules and uses the results to calculate the activity of the corresponding genes. Because all physiological responses are started by, and directed by, genes switching on or off, this method can be highly accurate and reliable. The history and actual condition of any plant, animal, or microorganism is reflected in the activity profile of its genes. Gene expression analysis, or transcriptomics, is performed using microarrays or biochips. On these microarrays, several thousand copied genes are present on a glass slide of a few square centimeters. The array is used to simultaneously detect the level of activity of each of the represented genes. Thus, the responses of the plant to any environmental trigger can be followed in a direct way. The information is used to select those genes that are most involved in controlling the trait of interest, in this case cold hardiness. Because microarray analysis is very expensive and not suitable for use in practice, NSure translates the selected set of indicator genes into a reliable and robust assay. A comparable technology has been used in medical diagnostics for several years, predominantly for making complex diagnoses such as tumor typing (Landers and others 2005; Modlich and others 2005). The bottleneck for using mRNA as a diagnostic tool in production and trade of agroproducts was the fact that RNA molecules are highly unstable. Taking a reliable sample required skilled personnel and hazardous chemicals or liquid nitrogen. The sampling procedure developed by NSure is based on FTA technology from Whatmann (Roy and Nassuth 2005), and circumvents these problems.

In 2006, NSure cold-tolerance assays were made available for Scots pine (*Pinus sylvestris*), Norway spruce (*Picea abies*), and European beech (*Fagus sylvatica*). The assay is based on the relative activity of 3 indicator genes that together provide enough information to give an estimate of the cold hardiness stage of the seedling. The corresponding genes dominate the process of hardiness development in all provenances studied and have a strong correlation with shoot electrolyte leakage (SEL) values (Joosen and others 2006; Balk and others 2007).

Douglas-fir Test

Because nurseries in British Columbia, Canada and the US Pacific Northwest region were interested in the technology, NSure developed a new assay aimed at one of the most economically important species in this region, Douglas-fir (*Pseudotsuga menziesii*). This new test was evaluated during the 2006 to 2007 season as part of a larger cold hardiness project with the Nursery Technology Cooperative (Oregon State University, Corvallis, Oregon). This project examined the relationships among cold hardiness development, thermoperiod (chilling hours), photoperiod, and calendar date in order to generate information to assist with nursery lifting and storage decisions. This paper will only discuss the results as they relate to the gene activity test. The full results will be published separately.

Material and Methods

Seedlings and Sampling Procedures

Douglas-fir seedlings from 3 stocktypes and 3 participating nurseries were used:

1) 2+0 bareroot (Webster Nursery, Olympia, Washington);
2) 1+0 bareroot for transplant (Lewis River Reforestation, Woodland, Washington);
3) PP21 outside-grown container (Microseed Nursery, Ridgefield, Washington).

Two seed sources (low/high elevation) within each stocktype were chosen for expected differences in hardiness:

1) 2+0 stocktype
   Low = lower Columbia (0 to 300 m [0 to 1000 ft])
   High = Yakama (900 to 1200 m [3000 to 4000 ft])
2) 1+0 stocktype
   Low = seed zone 262 (150 m [500 ft])
   High = seed zone 262 (610 m [2000 ft])
3) PP21 container stocktype
   Low = seed zone 051 (300 m [1000 ft]); coastal
   High = seed zone 452 (670 m [2200 ft]); Clackamas

Seedlings were lifted on 5 lift dates in 2006 (16 October, 30 October, 13 November, 27 November, 11 December) using standard nursery procedures. At each lifting date, a sample of 60 seedlings was assessed for cold hardiness by the whole plant freeze test (WPFT) and the NSure test.

Cold Tolerance Testing

Seedlings in the WPFT were divided into 4 groups, each frozen to a specific target temperature in a programmable chest freezer. After freezing, seedlings were placed into a greenhouse with optimal growth conditions for 6 days, and then assessed for foliar, bud, and cambial damage to estimate the LT_{10} or LT_{50} (lethal temperature to 10% or 50% of the seedlings, respectively). The NSure test was conducted on needles and buds collected from the same seedlings used for the WPFT test. The tissue was processed according to the sampling protocol provided with the NSure sampling kit. For each seedling group on each test date, buds and needles were sampled from 15 seedlings. Buds and needles were separately transferred to a vial containing extraction fluid, and a pestle was used to crush the tissue in the fluid for about 1 minute. Subsequently, 1 drop of extract was transferred to an FTA card and allowed to dry at room temperature. There were 3 replications for every sample date and seedling group. Samples were then sent to The Netherlands for analyses. NSure measured the level of expression of 3 genes for which the activity is expected to change in relation to cold hardiness development. Two should show increased activity, and 1 should show decreased activity. In addition, 3 control genes for which the activity was expected to remain constant were measured. Expression levels were measured using real-time RT PCR and specific primers designed by NSure, according to standard protocols. The corresponding hardiness status was calculated using models derived from Scots pine and Norway spruce. The Douglas-fir specific indicator genes were isolated based on their homology with the corresponding Scots pine genes.

Selection of Douglas-fir Specific Indicator Genes

Three of the most reliable indicator genes used in the Scots pine assay were selected for the Douglas-fir test. The corresponding Douglas-fir genes were identified through data mining in a public database (URL: http://www.ncbi.nlm.nih.gov). Because the provenance used in the database was not the same as the ones used in this trial, we decided to further maximize the homology by isolating the gene fragments specific for a common Oregon seed source. Therefore, specific PCR primers were constructed based on the sequence from the database, and the corresponding gene fragments were isolated from the cDNA of the

seedlings. Clones obtained were sequenced and subsequently identified using BLAST analysis. Specific primers were designed, based on the new specific sequence information, and those were used for expression analysis.

The differences in hardiness development are reflected in the development of gene expression of the 3 indicator genes. All gene expression values are relative to the expression of control genes that remain a constant activity over time. This method ensures that different samples can be compared even though the absolute levels of mRNA may not be the same, for example, due to differences in the extraction procedure.

Results

Cold Hardiness Development

As expected, the 6 provenances showed variations in cold tolerance development associated with the geographical source from which the seeds originated. Figure 1 shows the development of cold hardiness (LT_{50}) of each seedlot through the fall months of 2006 as determined with WPFT.

The results of the NSure cold tolerance test are shown in Table 1. The table gives an overview of all data and indicates the NSure hardiness stage that is commercially used. From the table, it is obvious that the patterns in cold tolerance that were detected through the WPFT correspond to that of the NSure assay on buds. The only exception was found to be the high elevation seed source grown at Webster Nursery (Yakama, 900 to 1200 m [3000 to 4000 ft]), which showed early entrance into NSure phase 3.

The expression pattern obtained from needles was less conclusive. This is partly due to the fact that, in some cases, no RNA could be extracted from the FTA Cards; in other cases, some of the indicators were not detectable at all. In cases where, technically, everything was correct, phase definition deviated regularly from that of the buds.

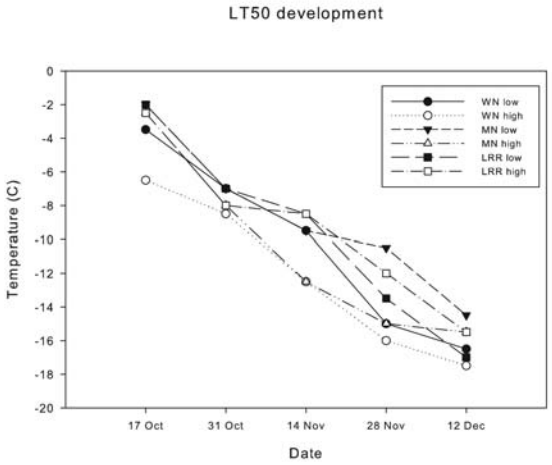

Figure 1. Development of LT_{50} temperatures over time for the 6 seedlots tested in the study. The graphs show the mean values of 3 measurements for each time point. WB = Webster Nursery; LRR = Lewis River Reforestation; MS = Microseed Nursery; high = high elevation seed source; low = low elevation seed source.

Discussion

Comparison of the gene expression profiles derived from bud tissue and the results of the WPFT showed a strong correlation between the 2 datasets. This indicates that the gene expression method is a good alternative for cold hardiness testing. Statistical analyses of the data allowed us to distinguish 3 stages of frost tolerance. These phases were shown to correlate with the LT values in the following way:

NSure phase 1: No frost tolerance observed.
NSure phase 2: LT_{50} value between -5 and -10 °C (23 to 14 °F); LT_{10} value between -1 and -5 °C (30 to 23 °F).
NSure phase 3: LT_{50} value below -10 °C (14 °F); LT_{10} value below -5 °C (23 °F).

This correlation remains true for all stocktypes and for seedlots from both high and low elevations, provided buds were used for the gene profiling. In the case of the high elevation seed source grown at Microseed Nursery (Clackamas), expression profiles seem to be even more consistent than LT profiles.

Table 1. Gene expression profiles and ColdNSure stages for *Pseudotsuga menziesii*. The table shows the gene expression levels relative to internal standards. The first portion gives the result for buds, and the second portion for needles, taken from the same seedlings. The right column indicates the corresponding ColdNSure phase: Phase 1 = no detectable sign of frost tolerance development; Phase 2 = start hardening; Phase 3 = fully hardened; ND = not detectable. WB = Webster Nursery; LRR = Lewis River Reforestation; MS = Microseed Nursery; HI = high elevation seed source; LO = low elevation seed source.

BUD TISSUE

Sampling date (2006)	Description	biological replicate	expression (relative to internal standard)				NSure phase
			PmD01	PmU01	PmU02	PmU01/PmD01	
12 October	LRR HI	1	nd	nd	nd		
		2	nd	nd	nd		
31 October	LRR HI	1	13.23	9.50	10.12	0.5	2
		2	68.56	10.86	6.90	2.7	2
14 November	LRR HI	1	14.04	7.06	9.01	1.0	2
		2	33.49	29.68	28.32	0.2	2
28 November	LRR HI	1	24.35	32.40	44.39	-0.4	3
		2	21.62	27.91	53.03	-0.4	3
12 December	LRR HI	1	26.43	115.43	90.89	-2.1	3
		2	20.46	98.29	150.51	-2.3	3
12 October	LRR LO	1	11.17	nd	3.01		1
		2	9.58	2.14	4.13	2.2	2
31 October	LRR LO	1	102.09	22.93	11.06	2.2	2
		2	79.56	78.98	53.44	0.0	2
14 November	LRR LO	1	91.51	68.34	34.38	0.4	2
		2	53.29	128.36	81.69	-1.3	3
28 November	LRR LO	1	41.14	111.69	96.81	-1.4	3
		2	108.95	566.49	253.23	-2.4	3
12 December	LRR LO	1	44.43	259.34	209.52	-2.5	3
		2	27.37	384.27	368.54	-3.8	3
12 October	MS HI	1	nd	9.22	3.27		1
		2	21.60	7.83	35.73	3.3	2
31 October	MS HI	1	49.06	42.24	12.85	0.6	2
		2	5.56	55.05	42.28	-1.2	2
14 November	MS HI	1	5.73	62.93	45.54	-1.3	2
		2	8.80	32.00	28.00	-0.4	2
28 November	MS HI	1	1.01	195.31	153.18	-3.7	3
		2	0.70	260.86	206.04	-4.2	3
12 December	MS HI	1	0.46	533.59	398.90	-4.9	3
		2	1.34	420.22	357.29	-3.1	3
12 October	MS LO	1	18.35	7.59	12.58	1.3	1
		2	17.70	6.73	10.31	1.4	1
31 October	MS LO	1	167.73	100.30	29.96	0.7	2
		2	110.75	61.88	28.64	0.8	2
14 November	MS LO	1	166.85	55.91	32.30	1.6	2
		2	135.26	108.72	68.80	0.3	2
28 November	MS LO	1	98.60	392.39	104.24	-2.0	3
		2	319.85	659.77	195.06	-1.0	3
12 December	MS LO	1	67.59	408.21	241.53	-2.6	3
		2	23.09	235.06	166.16	-3.3	3

BUD TISSUE

Sampling date (2006) phase	Description	biological replicate	expression (relative to internal standard)				NSure
			PmD01	PmU01	PmU02	PmU01/PmD01	
12 October	WB HI	1	10.58	11.00	2.84	-0.1	2
		2	4.64	11.35	1.07	-1.3	2
31 October	WB HI	1	126.51	691.73	397.56	-2.5	3
		2	554.41	2738.83	1241.91	-2.3	3
14 November	WB HI	1	179.05	2998.76	1498.32	-4.1	3
		2	335.24	3435.51	1629.01	-3.4	3
28 November	WB HI	1	57.87	2031.05	1279.30	-5.1	3
		2	38.41	1397.53	1070.07	-5.2	3
12 December	WB HI	1	16.55	438.24	399.44	-4.7	3
		2	31.42	937.10	1038.56	-4.9	3
12 October	WB LO	1	75.19	31.16	27.72	1.3	1
		2	64.50	15.01	14.62	2.1	1
31 October	WB LO	1	141.60	777.06	374.28	-2.5	2
		2	32.88	42.18	36.89	-0.4	2
14 November	WB LO	1	60.00	170.22	131.15	-1.5	2
		2	69.73	403.09	216.29	-2.5	2
28 November	WB LO	1	11.26	933.25	987.79	-6.4	3
		2	19.75	761.43	903.82	-5.3	3
12 December	WB LO	1	25.27	754.09	608.63	-4.9	3
		2	41.73	845.72	623.06	-4.3	3

NEEDLES

Sampling date phase	Description	biological replicate	expression (relative to internal standard)				NSure
			PmD01	PmU01	PmU02	PmU01/PmD01	
12 October	LRR HI	1	18.99	6.71	6.62	1.5	1
		2	109.88	28.03	45.29	2.0	1
31 October	LRR HI	1	12.21	40.69	21.96	-1.7	1
		2	35.73	135.04	82.90	-1.9	2
14 November	LRR HI	1	15.20	215.85	135.91	-3.8	2
		2	15.56	157.32	91.49	-3.3	2
28 November	LRR HI	1	47.11	1492.80	866.13	-5.0	3
		2	26.06	2621.27	960.06	-6.7	3
12 December	LRR HI	1	13.21	382.93	402.73	-4.9	3
		2	5.71	694.91	877.97	-6.9	3
12 October	LRR LO	1	17.19	30.48	30.97	-0.8	1
		2	32.88	10.07	nd	1.7	1

Table 1 [continued]. Gene expression profiles and ColdNSure stages for *Pseudotsuga menziesii*. The table shows the gene expression levels relative to internal standards. The first portion gives the result for buds, and the second portion for needles, taken from the same seedlings. The right column indicates the corresponding ColdNSure phase: Phase 1 = no detectable sign of frost tolerance development; Phase 2 = start hardening; Phase 3 = fully hardened; ND = not detectable. WB = Webster Nursery; LRR = Lewis River Reforestation; MS = Microseed Nursery; HI = high elevation seed source; LO = low elevation seed source.

NEEDLES

Sampling date	Description	biological replicate	expression (relative to internal standard)				NSure phase
			PmD01	PmU01	PmU02	PmU01/PmD01	
31 October	LRR LO	1					
		2	31.55	76.84	47.43	-1.3	2
14 November	LRR LO	1	2.77	25.15	19.67	-3.2	2
		2	6.50	17.48	18.92	-1.4	2
28 November	LRR LO	1	nd	356.83	253.40		
		2	nd	180.00	204.57		
12 December	LRR LO	1	nd	93.76	105.53		
		2	4.02	233.77	156.26	-5.9	3
12 October	MS HI	1					
		2	12.00	1.71	1.03	2.8	1
31 October	MS HI	1	7.58	61.16	48.81	-3.0	3
		2	8.55	100.43	37.97	-3.6	3
14 November	MS HI	1		208.86	73.59		
		2		250.06	123.04		
28 November	MS HI	1	6.03	76.08	50.85	-3.7	3
		2	3.92	185.55	104.08	-5.6	3
12 December	MS HI	1	21.58	448.85	204.75	-4.4	3
		2	41.14	515.26	329.41	-3.6	3
12 October	MS LO	1	2.58	38.19	34.54	-3.9	2
		2					
31 October	MS LO	1	4.64	332.30	166.03	-6.2	3
		2	33.57	983.82	597.40	-4.9	3
14 November	MS LO	1	nd	286.92	231.80		
		2	18.94	511.63	306.63	-4.8	3
28 November	MS LO	1	2.52	627.87	333.71	-8.0	3
		2					
12 December	MS LO	1	nd	946.59	440.43		
		2				-6.5	3
12 October	WB HI	1	4.35	11.55	12.66	-1.4	1
		2	9.64	10.13	12.14	-0.1	1
31 October	WB HI	1	7.46	57.06	35.47	-2.9	2
		2	14.30	125.31	96.61	-3.1	2
14 November	WB HI	1	0.70	60.85	31.40	-6.4	2
		2	13.25	567.25	340.06	-5.4	3
28 November	WB HI	1	8.83	1544.77	763.51	-7.5	3
		2	14.27	1148.79	553.57	-6.3	3
12 December	WB HI	1	1.83	134.92	74.30	-6.2	2
		2	17.67	272.88	186.83	-3.9	2

Sampling date phase	Description	biological replicate	expression (relative to internal standard)				NSure
			PmD01	PmU01	PmU02	PmU01/PmD01	
12 October	WB LO	1	1.55	22.37	41.59	-3.9	2
		2	6.44	69.22	87.83	-3.4	2
31 October	WB LO	1	5.83	280.79	320.62	-5.6	3
		2	36.10	182.81	79.50	-2.3	3
14 November	WB LO	1	41.50	299.60	237.98	-2.9	3
		2	64.88	791.65	nd	-3.6	3
28 November	WB LO	1	11.75	1193.79	1577.10	-6.7	3
		2	227.39	13272.08	7774.97	-5.9	3
12 December	WB LO	1	12.32	270.04	307.02	-4.5	3
		2	42.08	269.81	306.43	-2.7	3

When needles were used, however, the correlation was poor. In contrast to previous findings with Norway spruce and Scots pine, this study indicates that Douglas-fir needles may not be as reliable for test material. One possible technical reason could be in cases where no good quality RNA is present on the FTA Cards. The tissue may be much more rigid, or may contain more inhibiting components which hamper extraction. This can also be a reason for the fact that, in other cases, indicators simply couldn't be measured. However, there may be a physiological explanation in cases where extraction proceeds properly, but phase definition deviates from that found for buds from the same seedling (for example, when frost tolerance levels in the needles are subject to fluctuation; Stralbiski 2007). Further research is required for this situation.

The fact that the expression profile of the same set of genes is indicative for cold tolerance in such diverse species as *Pinus sylvestris*, *Fagus sylvatica*, and *Pseudotsuga menziesii* suggests that the NSure test measures a biological process that was highly conserved in evolution. Usually this indicates that the process concerned is essential to plant survival. In practical terms, it means that measuring the activity of these conserved genes will give a highly accurate indication of the cold hardiness development, since they are likely the basis of the physiological process which occurs.

The big advantage of the NSure test is that seedlings do not have to be transported to a test laboratory. The samples can be taken and stabilized on site. Furthermore, the result time of the test is short, just a few days, which makes it suitable for integration into nursery logistics.

Conclusions

The strong correlation between the 2 cold hardiness tests in Douglas-fir seedlings, combined with the short analysis time required, suggests that NSure is highly suitable for implementation as a nursery management decision support tool.

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